"The journey of a thousand miles begins with one step"

Lao Tzu

Zinc status assessment and the impact of dietary zinc on claw quality in pigs

Miriam M.J. van Riet

FACULTY OF **VETERINARY MEDICINE**
approved by EAEVE

Thesis committee

Promotors
Prof. dr. S. Millet
Institute for Agricultural and Fisheries Science (ILVO)
Department of Animal Sciences
&
Faculty of Veterinary Medicine, Ghent University
Laboratory of Animal Nutrition, Department of Nutrition, Genetics, and Ethology

Prof. dr. ir. G.P.J. Janssens
Faculty of Veterinary Medicine, Ghent University
Laboratory of Animal Nutrition, Department of Nutrition, Genetics, and Ethology

Other members
Prof. dr. A. Van Soom, Faculty of Veterinary Medicine, Ghent University
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Prof. dr. D. Maes, Faculty of Veterinary Medicine, Ghent University
Prof. dr. F.A.M. Tuyttens, ILVO & Faculty of Veterinary Medicine, Ghent University

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Dissertation submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Veterinary Sciences, Faculty of Veterinary Medicine, Ghent University

Miriam M.J. van Riet

Institute for Agricultural and Fisheries Science (ILVO)
Department of Animal Sciences
&
Faculty of Veterinary Medicine, Ghent University
Laboratory of Animal Nutrition, Department of Nutrition, Genetics, and Ethology

Promotors: Prof. dr. S. Millet and Prof. dr. ir. G.P.J. Janssens

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Faculty of Veterinary Medicine, Ghent University Laboratory of Animal Nutrition Department of Nutrition, Genetics, and Ethology

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Preface

Lameness is one of the main reasons for culling sows before they reach their full breeding potential and it negatively affects the welfare of pigs and farm profitability. Additionally, within the European Union, gravid sows need to be housed in groups from 28 days after insemination until one week before parturition. This seems to increase the presence of lameness.

Lameness may have both infectious and non-infectious origins. The most frequently observed non-infectious causes are osteochondrosis, arthritis, and claw lesions, affecting the bone, articular cartilage and claw quality. Housing condition, management system, genetics and nutrition are important factors related to the occurrence of these non-infectious causes for lameness.

Within this thesis, the focus is on nutrition and more in particular on zinc (Zn). However, to understand the whole concept, a broad overview of the influence of diet composition on the quality of bone, articular cartilage and claw will be provided first. From this overview, we will emphasise on the normal and pathological claw and on the different aspects that are important for the metabolism of Zn. When looking into claw anatomy, types of claw lesions, process of horn production and influence of nutrients, the function of Zn as essential micromineral with a postulated role in claw quality becomes apparent.

Zinc is required in many processes within the body and the metabolism is tightly regulated to maintain homeostasis. The complexity of Zn metabolism is illustrated by showing the effect of both insufficient and excessive dietary Zn intake, and influence of other minerals. The need to limit Zn provision in the diet originates from its ecotoxicity. However, to decrease dietary concentrations, there is a need to determine adequate Zn requirements.

In order to achieve this goal, we first identified appropriate sensitive and/or specific Zn status biomarkers through a literature review (Chapter 3). The most common biomarkers described in human studies are evaluated on its suitability for production animals, including the pig. Based on this evaluation, we assessed the impact of reproductive phase on these biomarkers (Chapter 4) and we studied the interaction between Zn and protein source on Zn status biomarkers and Zn bioavailability in a period with declining plasma Zn concentrations (Chapter 5).

Finally, in the concluding part of this thesis (Chapter 6) we describe two experiments on the impact of dietary Zn concentration on Zn status biomarkers and claw quality in piglets and sows. The main findings on Zn status biomarkers and claw quality are then discussed in a general discussion (Chapter 7).

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List of abbreviations

AC	Articular cartilage
ADF	Acid Detergent fiber
ADG	Average daily gain
ADL	Acid Detergent Lignin
AIA	Acid insoluble ash
ALA	Alpha linolenic acid
ALP	Alkaline Phosphatase
Arg	Arginine
ATP	Adenosine triphosphate
Avg	Average
BCS	Body condition score
BF	Backfat thickness
BMD	Bone mineral density
BW	Bodyweight
C	Claw
Ca	Calcium
CC	Claw conformation measurements
Cd	Cadmium
Cl	Chloride
CL	Claw lesion scoring
Co	Cobalt
CP	Crude protein
Cr	Chromium
Cu	Copper
CV	Coefficient of variability
CVB	Centraal Veevoederbureau
d	Claw digits
DD	Digital dermatitis
dEB	Dietary electrolyte balance
DHA	Docosahexaenoic acid
DJD	Degenerative joint disease
DM	Dry matter
ECM	Extracellular matrix
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentaenoic acid
F	Fluorine
Fe	Iron
FFDM	Fat free dry matter
Fl	Floor type
G	Horn growth
GAG	Glycosaminoglycans
GTF	Glucose tolerance factor
GW	Horn growth and wear
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HCt	Haematocrit level
HF	Hydrolysed feather meal
His	Histidine
HNO_3	Nitric acid
HRP	Horseradish Peroxidase
I	Iodine
ICS	Intercellular cementing substance
ID	Ileal digestible
IDD	Interdigital dermatitis
IGF-1	Insulin-like growth factors 1
Ile	Isoleucine
ISO	International organisation for standardisation
IZ	Inorganic Zn
IZINCG	International Zn nutrition consultative group
K	Potassium
$KHCO_3$	Potassium bicarbonate
KOH	Potassium hydroxide
L	Lateral
Leu	Leucine
LOG	Logarithm
Lys	Lysine
M	Medial
MCC	Mechanical claw characteristics
Met	Methionine
Mg	Magnesium
MJ	Mega Joule
MMP	Matrix metalloproteinase
Mn	Manganese
Mo	Molybdenum
mo	Months
MPa	Megapascal
MRE	Metal response element
mRNA	Messenger RNA
MT	Metallothionein
MTF	Metal-binding transcription factor
MTL	Maximum tolerance level
Na	Sodium
NDF	Neutral Detergent fiber
NEv	Net energy for pigs
NIRS	Near-infrared spectroscopy
NO	Nitric oxide
NRC	National Research Council
OC	Osteochondrosis
OZ	Organic Zn
P	Phosphorus
P	P-value
P2	Position 2 for backfat thickness
PG	Proteoglycan
Phe	Phenylalanine

PUFA	Polyunsaturated fatty acids
QQ-plot	Probability plot
RNA	Ribonucleic acid
S	Sulphur
SAMe	S-adenosylmethionine
SAS	Statistical analysis system
SB	Soybean meal
SCC	Somatic cell count
SD	Standard deviations
Se	Selenium
SE	Standard error
SeCys	Selenocysteine
SeMet	Selenomethionine
Si	Silicium
SFA	Saturated fatty acids
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TG	Epidermal transglutaminase
Thr	Threonine
Trp	Tryptophan
tVAS	Tagged visual analogue scale
Val	Valine
vP	Digestible Phosphorus
W	Horn wear
WHO	World health organisation
ZIP	Zrt- and Irt-like proteins
Zn	Zinc
ZnT	Zn transporter protein families
+Zn	Zn supplementation
-Zn	Non-Zn supplementation
ZnO	Zinc oxide
1,25-$(OH)_2D_3$	Calcitriol

Chapter 1

General introduction

Adapted from invited review: Impact of nutrition on lameness and claw health in sows.

M.M.J. van Riet, S. Millet, M. Aluwé, and G.P.J. Janssens.

Special issue Livestock Science (2013) 156, 24-35.

Introduction

The prevalence of lameness in Western European sows is estimated at 10 to 15% (Boyle *et al.*, 2010; Engblom *et al.*, 2007), whereas the prevalence of sows with one or more claw lesions varies between 50 and 100% (Anil *et al.*, 2007; Knauer *et al.*, 2007).

Lameness and claw lesions have considerable consequences for animal welfare and farm profitability. The welfare issues are related to pain associated with lameness and limitations in the ability to perform social behaviours or explore the environment (Anil *et al.*, 2005; Johnson, 2010; Heinonen *et al.*, 2013). Sow lameness and claw lesions also have considerable economic consequences. The culling rate due to lameness and claw lesions is estimated between 6 and 35% (Anil, 2011, Pluym *et al.*, 2011, 2013a,b; Heinonen *et al.*, 2013). A lame sow generally produces fewer than 3 litters (Anil *et al.*, 2009; Pluym *et al.*, 2013b), whereas a non-lame sow produces 3.5 litters before removal from the herd (Vestergaard *et al.*, 2004). Lameness is therefore one of the main reasons for culling young sows before achieving their full breeding potential (Anil *et al.*, 2009). Piglet mortality is also higher for lame sows (27.7%) than non-lame sows (12.4%) (Anil *et al.*, 2009). Additional economic consequences are increased labour, higher diagnostic and treatment costs, lost genetic premium, and decreased slaughter value (Anil *et al.*, 2009; Heinonen *et al.*, 2013).

Lameness has both infectious and non-infectious causes (Wendt, 2011). The most frequently observed causes for sow lameness are osteochondrosis (OC), arthritis, and claw lesions (Bradley, 2010; Wendt, 2011). These disorders affect the bone, articular cartilage and horn quality, respectively (Figure 1.1). Nutrition plays an important role in lameness, especially with regard to the diet composition, feed intake and feeding management (Bradley, 2010; Crenshaw *et al.*, 2010; Knauer *et al.*, 2007). Still, in most cases, the occurrence of lameness and claw lesions does not depend on a single predisposing factor such as malnutrition, but is inextricably interconnected with other factors (Anil *et al.*, 2007; Muelling, 2009). However, previous research focussed mainly on single factors and its impact on lameness, whereas the relations between factors are not specified.

This general introduction focusses on the importance of diet composition in the occurrence of lameness with a non-infectious origin in pigs. When information for pigs is lacking, studies in humans and other species are included for comparison. We will consecutively focus on the three main locomotory structures affected during lameness that are also influenced by diet composition: bone, articular cartilage and horn. Muscle weakness or central nervous system abnormalities may also result in symptoms of lameness but are outside the scope of this introduction (see supplemental information, Table 1.2).

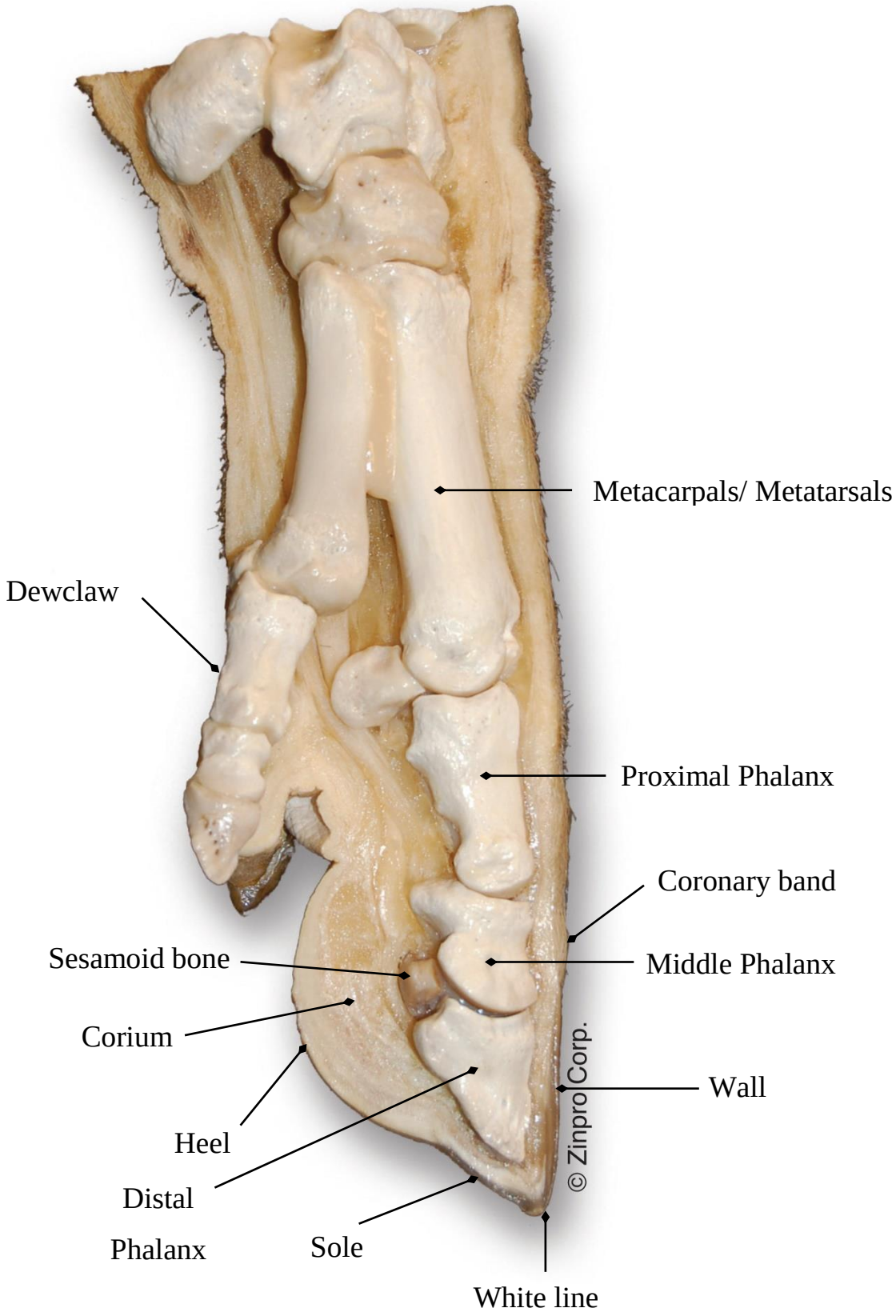

Figure 1.1. The sows' lower leg, illustrating tissues and structures which may influence bone, articular cartilage, and horn production (Ossent, 2010) (Reprinted with permission of © ZINPRO corporation, Eden Prairie, MN, USA, www.zinpro.com).

Bone and bone remodelling

Bones support and protect various organs, permit locomotion, contains haematopoietic tissue which produce red and white blood cells, provide structure to the body, maintain mineral homeostasis and acid-base balance, and store minerals and fat (Clarke, 2008; Ralston, 2009).

Bone is made up of bone tissue, bone marrow, periosteum, endosteum, nerves, and blood vessels. Bone tissue contains an extracellular matrix (ECM), with an organic and inorganic component. The organic matrix ensures flexibility and elasticity, whereas bone minerals provide strength and mechanical rigidity to the bone (Clarke, 2008; Ralston, 2009). The organic matrix consist of collagen (ossein) structured, via pyridinium cross-links, in fibrils, and small amounts of mucopolysaccharide (glycosaminoglycans- GAG) (Clarke, 2008; Ralston, 2009). The inorganic matrix contains hydroxyapatite crystals and small amounts of carbonate, citrate, Mg, Na, K, Cl, and F (Clarke, 2008). Hydroxyapatite crystals are formed by osteoblasts, which combine collagen with calcium (Ca) and phosphorus (P) to form these crystals. The formation of hydroxyapatite crystals is necessary for overall bone size and bone width. The formation of hydroxyapatite crystals and thereby the regulation and deposition of bone minerals is regulated by Ca- and P-binding proteins, such as alkaline phosphatase (ALP) and osteocalcin. This is facilitated by ECM vesicles, which contain proteins and a complex of phospholipids, Ca, and inorganic P in bone (the same as in calcifying cartilage) (Clarke, 2008).

During life, bone undergoes longitudinal growth as well as modelling and remodelling. During longitudinal growth, which occurs at the growth plates, cartilage proliferates and subsequently mineralises to form new primary bone. In bone modelling, the overall bone shape changes as a result of mechanical forces and physiological influences (Clarke, 2008). Bone remodelling occurs throughout life (Ralston, 2009), but is most common at aging. The purpose of this continuous process is to renew bone and to maintain the bone mineral content and bone strength.

Bone remodelling involves four phases (Figure 1.2). During the **activation** (quiescence) phase (1), preosteoclasts are formed, which become osteoclasts. These cells resorb bone in the osteoclast-mediated **resorption** phase (2). This is regulated by receptor activators such as vitamin D and calcitonin (Clarke, 2008). Resorption also includes the secretion of acids (carbonic, hydrochloric and lactic acid) and proteolytic enzymes through a membrane called the ruffled border in order to digest the organic matrix (Clarke, 2008; Goff, 2010; Ralston, 2009). The **transition** (reversal) (3) between bone resorption and **formation** (4) results in syntheses of the new organic matrix and regulation of matrix mineralisation by the osteoblasts. Small vesicles are released which contain Ca and P which will destroy mineralisation inhibitors like proteoglycan (PG) (Clarke, 2008). During the final step of formation, osteoblasts are subjected to apoptosis (programmed cell death) to

become osteocytes or bone-lining cells (Clarke, 2008; Ralston, 2009). The bone-lining cells may regulate the mineral flux of the bone extracellular fluid, acting as a blood-bone barrier. These cells can differentiate again to become osteoblasts during mechanical load and exposure of parathyroid hormones (Clarke, 2008).

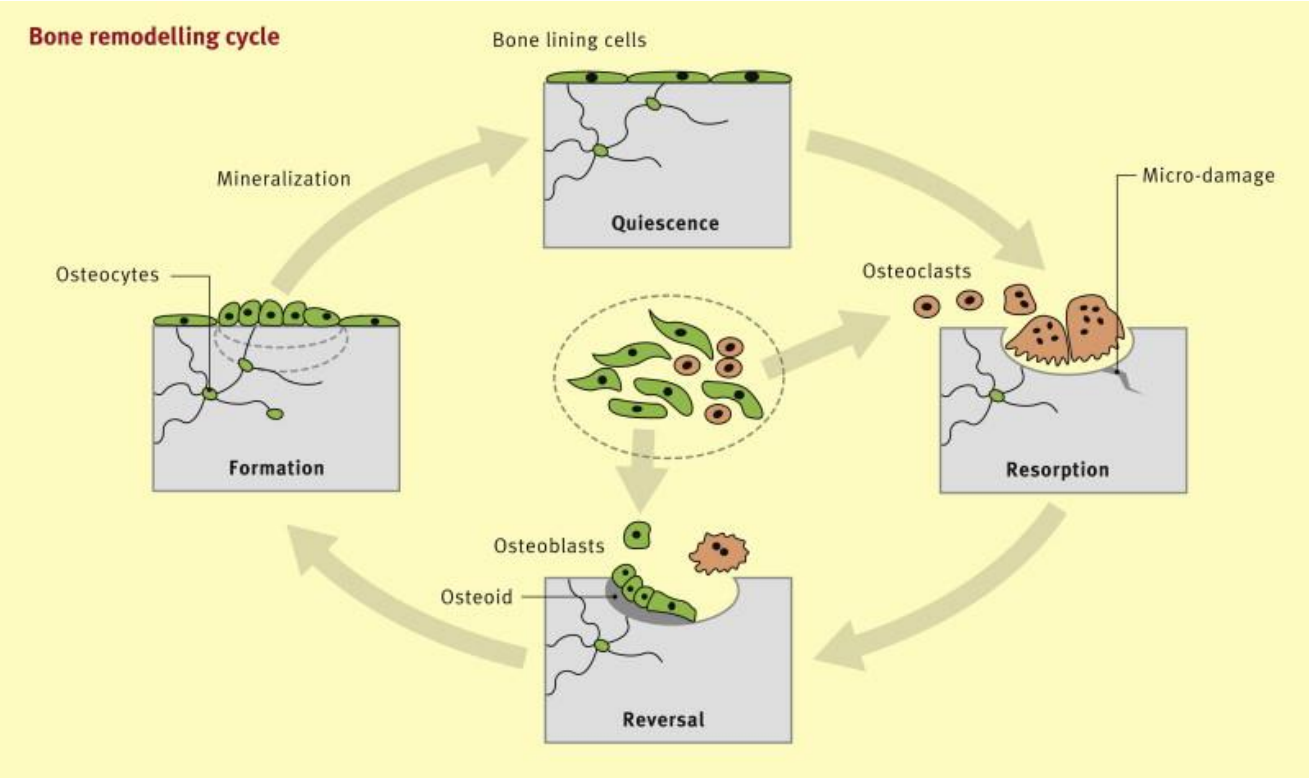

Figure 1.2. The continuous bone remodelling process to ensure optimal bone quality. Bone remodelling represents four succeeding phases: activation (quiescence), resorption, transition (reversal) and formation. These processes are required for bone replacement during lifetime (Clarke, 2008; Goff, 2010: Ralston, 2009) (Reprinted from Medicine, Volume 37, Ralston, S.H., Bone structure and metabolism, pp. 471, Copyright 2009, with permission from Elsevier).

Bone formation and resorption are tightly coupled during bone remodelling (Clarke, 2008) and have an equal rate under normal conditions (Goff, 2010). This leads to a continuous interchange of Ca and P in bone, in the blood supply and in other body parts. Bone remodelling is therefore essential to maintain bone quality. It can be locally triggered during microcrack formation or osteocyte apoptosis (Clarke, 2008).

Nutrition plays an important role in the development and maintenance of bone, because the bone structure and content varies according to nutrition (Bonjour, 2005). If the mineral supply or concentration is not appropriate, the bone quality is subsequently affected. This may result in an increased risk for lameness, because the bone protein matrix only calcifies when appropriate mineral concentrations are present (McDowell, 2003). Lactation may also affect bone quality, because mineralisation exceeds deposition during this physiological phase. The bone can become

weak, porous, deformed, and even fractured, as in case of osteoporosis, which is prevalent in breeding sows (Crenshaw, 2006). These types of damage to the bone structure can occur rapidly, before the sow can rebuild her bone integrity during the next gestation period (Bradley, 2010).

Nutritional impact on bone quality

Carbohydrates

Sound scientific evidence for the effect of carbohydrates is lacking for pigs. According to some authors, increased dietary fibre content decreases Ca absorption in humans (Heaney *et al.,* 1991), which may negatively affect bone mass. One study in humans found a negative correlation between fibre intake and bone density, while others found no association (Leuenberger *et al.,* 1989; Yano *et al.,* 1985). According to Reid and New (1997), these contradictory results might be explained by the assumption that dietary phytate content rather than the fibre content decreases Ca absorption, because phytate can bind to Ca, Cu and Zn thereby forming indigestible complexes (Revy *et al.,* 2004; Jongbloed *et al.,* 2013). Furthermore, lactose may have a positive effect on bone health as a result of increased Ca absorption (Heaney, 2009). However, the effect of dairy products on bone health is not understood and most studies in humans did not show an effect (Weinsier and Krumdieck, 2000), except for one interesting study comparing food based Ca (mainly low-fat cheese) with supplements of Ca or vitamin D. This study showed greater cortical thickness of the tibia and BMD when humans consumed food based Ca (Cheng *et al.,* 2005; Heaney, 2009).

Proteins and amino acids

The effect of dietary proteins on bone remodelling remains unclear for pigs. The impact of proteins on bone is dependent on protein source, dietary protein levels, Ca intake, and the dietary acid/base balance (Heaney and Layman, 2008). The effect of dietary protein levels are contradictory. Dietary protein content had no significant effects on bone mineralisation of the third metatarsals in gilts as assessed by bone strength and mineral characteristics (Slevin *et al.,* 2001). Also in humans, a meta-analysis did not confirm an effect of dietary protein levels on bone health and related bone fractures (Darling *et al.,* 2009). Other studies on humans found detrimental effects of low protein diets, because it decreases bone mass and bone strength (Bonjour, 2005; Heaney and Layman, 2008). High dietary protein diets have been reported to decrease Ca absorption and increase urinary Ca excretion in humans (Lutz, 1984), as well as to increase Ca absorption (Kerstetter *et al.,* 2003). The effect of protein level may differ between bones due to their function, because a slightly positive effect on lumbar spine bone mineral density (BMD) in humans was found during protein

supplementation, irrespective of protein source. However, this did not result in lower bone fracture rates (Darling *et al.*, 2009).

Further research is necessary to determine the effect of protein level, protein source and the pathophysiological hypothesis of increased acid production on Ca absorption (*i.e.* the effect of protein on Ca absorption is dependent on the dietary acid/base balance. If the diet contains a high proportion of acid forming ingredients, bone loss may be attributable to the mobilisation of mineral salts from bone tissue to balance the generated endogenous acids) (Heaney and Layman, 2008). Nevertheless, we postulate that dietary proteins are required for bone quality, because the cross-linkage of collagen during proteolysis (which occurs during bone remodelling) involves posttranslational modifications of amino acids, which cannot be reutilised to form a new organic matrix (Heaney and Layman, 2008). An adequate protein supply is therefore necessary. Furthermore, proteins enhance insulin-like growth factors 1 (IGF-1), which has a positive effect on bone formation.

Lipids

Dietary lipid content and type influence bone quality. Saturated fatty acids (SFA) are negatively associated with bone mineral density (BMD) and bone health in humans. Bone health can be improved by lowering dietary SFA content and increasing dietary polyunsaturated fatty acids (PUFA) content (Corwin *et al.*, 2006). The observed changes in bone quality can be due to alterations in lipid oxidation, osteoblast formation, Ca absorption, collagen content, collagen cross-linkage, and prostaglandin synthesis (Corwin *et al.*, 2006; Liu *et al.*, 2004). It has also been observed that bone mass of neonatal pigs increases when their diet includes PUFA (Blanaru *et al.*, 2004; Crenshaw, 2006).

Minerals

Macrominerals

Calcium (Ca) and phosphorus (P) are essential to maintain a high bone quality in both growing and breeding pigs. We discuss Ca and P together, because they are homeostatically regulated and the dietary Ca:P ratio should remain constant, with a minimum of 1:1 to maintain adequate P absorption (McDowell, 2003; Jacela *et al.*, 2010; NRC, 2012). However, the symptoms of deficiency of each mineral differ slightly. Under Ca deficiency, the formation of normal osteoids does not occur or osteoids are replaced by fibrous tissue. The growth plate is weakened and widened and growth is retarded in growing animals (Goff, 2010; McDowell, 2003; Suttle, 2010). Prolonged Ca deficiency results in lameness (Suttle, 2010). The trabecular bones, which are lowest

in ash content, are first affected during Ca deficiency and alterations are found in the total mineral content rather than in the proportion of minerals in the remaining ash (Suttle, 2010). In the case of P deficiency, the osteoids are formed normally but are not mineralised. Phosphorus deficiency also results in poor growth rate in growing animals (Goff, 2010; McDowell, 2003; Suttle, 2010). Both Ca and P deficiencies result in rickets in growing pigs (*i.e.* disturbed bone formation as a consequence of defective mineralisation of the cartilage matrix at the sites of endochondral ossification and newly formed osteoid) and osteomalacia in older sows (*i.e.* disturbed bone formation as a consequence of defective mineralisation of the osteoid formed during bone remodelling) (Suttle, 2010; Craig *et al.,* 2015).

Adequate dietary Ca and P intake is important, however, the intake is influenced by the physiological Ca and P requirements. These requirements are highest at the end of gestation and during lactation (McDowell, 2003). The sow's body reacts to this by uncoupling bone formation from bone resorption (Goff, 2010; NRC, 2012), which contributes to an overall loss of bone mass. The osteoclasts resorb bone and osteoblast movement is stagnated. Lactating sows receiving insufficient Ca and P levels during lactation can develop hypocalcemia and bone fractures, which typically take place in the third to fourth week of lactation when the milk production is high. Especially the axial skeleton bones are sensitive to this demineralisation process, while long bones and skeletal extremities are less affected (Goff, 2010).

Another factor important for adequate Ca and P intake is availability. Calcium has a relatively high availability, whereas P availability depends on the source. The addition of phytase to the diet has a beneficial effect on P absorption, because it increases P availability and decreases faecal P excretion (Rodehutscord *et al.,* 1999). This results in a higher Ca and P content, which increases bone ash, BMD and the breaking strength of the metatarsal and tibia bones (Bühler *et al.,* 2010).

To our knowledge, the relationship between sodium (Na) intake and bone strength has not been investigated in sows. Sodium has to be present in the lumen of the small intestine for absorption of sugars and amino acids, but it also plays a role in the absorption of protein by bones (Grim, 1980). Sodium deficiency results in decreased protein and energy utilisation (McDowell, 2003) which alters the protein and basic amino acid metabolism. However, in a short-term experiment with growing pigs (three weeks), the dietary electrolyte balance (dEB) did not affect bone mineral content gain, bone-breaking strength, bone ash, percentage of bone ash and Ca and P balance (Budde and Crenshaw, 2003).

Potassium (K) has a beneficial effect on mineral balance and skeletal metabolism but the effect of K on sow lameness has not been studied. In humans, $KHCO_3$ improves Ca balance and bone formation

but reduces bone resorption (Sebastian *et al.,* 1994). Therefore, the dietary K intake may influence bone quality and thereby influence the occurrence of lameness.

Magnesium (Mg) is important for bone integrity. It may be even as important as Ca, because Mg is required to convert vitamin D into its active form calcitriol (1,25-$(OH)_2$D3), which is necessary for Ca absorption. Magnesium also stimulates calcitonin and regulates parathyroid hormone, which stimulate osteoclasts to reabsorb calcified bone (Maher, 1999; Zofková and Kancheva, 1995). In humans, the dietary Mg intake directly affects bone Mg content (Aydin *et al.,* 2009). Magnesium deficiency severely reduces oxidative phosphorylation and increases bone resorption in humans (Manicourt *et al.,* 1981), whereas excessive Mg supplementation suppresses bone turnover. In some studies, the bone mineral density (BMD) is also increased during Mg supplementation, but other studies did not find effects in osteoporotic women (Aydin *et al.,* 2009). For sows, the effect of dietary Mg intake on bone quality and occurrence of lameness need to be clarified in order to provide updated Mg requirements in modern sow breeds.

Microminerals

The dietary zinc (Zn) intake is positively correlated with bone mass in humans (Bouglé *et al.,* 2004; Ovesen *et al.,* 2009). It influences IGF-1 and osteoblast activity, as well as the activity of 1,25$(OH)_2$D3, parathyroid hormone and alkaline phosphatase (ALP) (Bouglé *et al.,* 2004; Yamaguchi, 1998). Alkaline phosphatase is important in the formation of hydroxyapatite crystals, because it regulates ordered mineral deposition (Clarke, 2008). Zinc is also involved in maintaining plasma vitamin A concentrations, which influence bone development (McDowell, 2003). Zinc is therefore important for bone quality. During Zn deficiency, bone size, bone strength, and bone growth are negatively affected (McDowell, 2003), and Zn deficiency also markedly reduces the synthesis and turnover of chondroitin sulphate and bone collagen by reducing the activity of the Zn-dependent metalloenzyme tibial collagenase as found in mice (Starcher *et al.,* 1980). Zinc supplementation increases bone ALP activity of the femoral diaphysis in rats (Yamaguchi and Yamaguchi, 1986). Furthermore, Zn supplementation to meet requirements with simultaneous Fe supplementation increased metacarpal bone strength and ash weight in young pigs, because if only Zn was supplemented Fe absorption decreased and Fe seems to be important for BMD (Veum *et al.,* 2009).

Copper (Cu) is important for bone formation and connective tissue development (Andrieu, 2008). Copper deficiency results in a marked reduced osteoblast activity and failure of bone deposition on the calcified cartilage matrix (McDowell, 2003).

Manganese (Mn) is essential to bone organic matrix development. The synthesis of glycosaminoglycans (GAG) of the organic matrix may be impaired during Mn deficiency, which has been related to the activation of glycosyltransferase. Glycosyltransferase catalyses chondroitin-sulphate chains of PG. The latter are essential for normal bone formation and thus maintain an adequate bone mineralisation and bone growth (Andrieu, 2008). Both too low and too high dietary Mn intakes may affect sow gait. Manganese deficiency results in shortened and thickened bones, lameness and enlarged hock joints in animals, whereas toxicity results in leg stiffness and stilted gait (McDowell, 2003).

Fluoride (F) has a high affinity for incorporation in bone and may prevent osteoporosis in humans (Li, 2003). The risk for lameness is more related to (excessive) F supplementation than to F deficiency, because F deficiency is uncommon. Fluoride supplemented to the diet of growing-finishing pigs resulted in increased serum alkaline phosphatase (ALP), bone F concentrations, and bone porosity, but decreased bone thickness (Li, 2003). The breaking strength was affected at high (650-970 ppm F) dietary F concentrations, but not when pigs were fed 290 and 580 ppm F (Kick *et al.*, 1933). The severity of the observed symptoms increases during toxicity, where F interferes with Ca metabolism, inhibits bone mineralisation and reduces the mechanical quality of bone crystals (Li, 2003).

Vitamins

Vitamin A is required for growth and reproduction and vitamin A deficiency results in bone abnormalities, including abnormal bone growth and poor bone development in poultry and dogs (Waldenstedt, 2006; Ahmadieh and Arabi, 2011). Toxicity may increase bone resorption and decrease bone formation, or a combination of both processes, accelerate bone remodelling (Ahmadieh and Arabi, 2011) and may induce hyperostosis (Romero *et al.*, 1996; Penniston and Tanumihardjo, 2006; Vyas and White, 2011). This results in bone loss and a high risk for spontaneous bone fractures (Binkley and Krueger, 2000; Ahmadieh and Arabi, 2011).

Vitamin B is associated with longitudinal bone growth and deficiency can lead to growth abnormalities. Dependent on type of vitamin B, deficiency interrupts bone metabolism and results in bone abnormalities (*e.g.* skeletal malformation, lowered BMD and osteoporosis along with increased fracture risk as observed in humans, poultry, rats and mice (Ahmadieh and Arabi, 2011; Dai and Koh, 2015)), confirming the importance of vitamin B in bone quality and occurrence of lameness. A major association was found between folate and bone mineralisation in humans regarding to lumbar spine bone mineral density (BMD) (Cagnacci *et al.*, 2003). The vitamin B requirements are increased at high protein concentrations, owing to interactions between vitamins

and proteins via transamination and/or deamination. This is especially true during marginal dietary vitamin B supply in poultry (Waldenstedt, 2006). More research is necessary to confirm the applicability of these findings for sows.

The impact of vitamin C on bone remodelling is not clear. It has been shown that vitamin C is involved in the synthesis of 1,25-$(OH)_2$D3 and collagen in poultry (Waldenstedt, 2006). Pigs have the capacity to synthesise sufficiently vitamin C from glucose (Frantz, 2006). Supplementation of vitamin C in poultry failed to show convincing evidence of a decrease in leg abnormalities and it remains unclear why some studies found positive effects, whereas others did not (Waldenstedt, 2006).

Vitamin D plays an important role in bone remodelling in pigs and other animals, because it controls Ca absorption, mobilisation, and accretion from bones. Vitamin D may also have a function in the repair of bone fractures (Goff, 2010). The active form of vitamin D (1,25-$(OH)_2$D3) maintains plasma Ca and P levels for mineralisation of unmineralised bone matrix (Clarke, 2008; Goff, 2010). The 1,25-$(OH)_2$D3 concentration is increased when the Ca and P requirements are high, resulting in increased levels of alkaline phosphatase (ALP), Ca-stimulated ATPase and phytase enzyme activities (Waldenstedt, 2006). During impaired absorption or liver hydroxylation of vitamin D, Ca and P availability is decreased, which may have negative consequences for bone.

Articular cartilage

The function of articular cartilage (AC) is to transmit the mechanical load to the underlying bone and to reduce friction during joint movement. Articular cartilage is a thin layer of chondrocytes embedded in a porous extracellular matrix (ECM). The ECM is saturated with interstitial fluid and mainly consists of collagen type II fibres enclosed in a proteoglycan (PG) matrix (van Turnhout, 2010; van Turnhout *et al.,* 2010). These collagen fibres are characterised by protein chains of glycine, lysine, cysteine, hydroxyproline, and hydroxylysine (Percival, 1997), whereas PGs are proteins with at least one negatively charged glycosaminoglycan (GAG), mainly aggrecan. These GAGs consist of repetitive chondroitin sulphate and keratin sulphate chains linked to hyaluronic acid (Figure 1.3) (Nakano *et al.,* 1987; van Turnhout *et al.,* 2010).

The main AC disorders are osteochondrosis (OC) and degenerative joint disease (DJD). Osteochondrosis is a developmental disturbance during endochondrial ossification that occurs in the physeal growth plate and the articular-epiphyseal cartilage of growing animals, including pigs (Crenshaw, 2006; Craig *et al.,* 2015). Endochondrial ossification occurs during skeletal growth in which AC change to calcified tissue (Crenshaw, 2006; Ytherus *et al.,* 2007; van Turnhout, 2010). Then, the AC layer becomes thinner, chondrocyte density decrease, collagen density and collagen

fibril diameter increase, fluid density decrease, and the GAG composition change (van Turnhout, 2010; van Turnhout *et al.*, 2010). Degenerative joint disease is a consequence of various forms of joint injury and reflects a progressive denegeration of one or more components of joints (*e.g.* AC, subchondrol bone and synovium) (Lascelles, 2010; Craig *et al.*, 2015). Degenerative joint disease is classified as primary (no apparent predisposing cause and generally occurs in older animals) and secondary (associated with an underlying abnormality in the joint or supportive tissue predisposing to premature degeneration) (Lascelles, 2010; Craig *et al.*, 2015). To prevent AC disorders, an optimal diffuse vascular nutrient and oxygen supply and waste product removal from the synovial fluid are essential since AC is an avascular tissue (van Turnhout *et al.*, 2010). During OC and DJD, the diffuse nutrient supply is disturbed. This results in a disturbed PG synthesis with a local PG depletion and less aggregated PG (Crenshaw, 2006; Nakano *et al.*, 1987). In particular, it decreases the molecular size of chondroitin sulphate and the ratio of chondroitin-4-sulphate to chondroitin-6-sulphate in lame pigs (Nakano *et al.*, 1987). The impact of nutrition is therefore related to collagen- and PG synthesis to ensure an optimal diffuse nutrient supply (Nakano *et al.*, 1987; Percival, 1997).

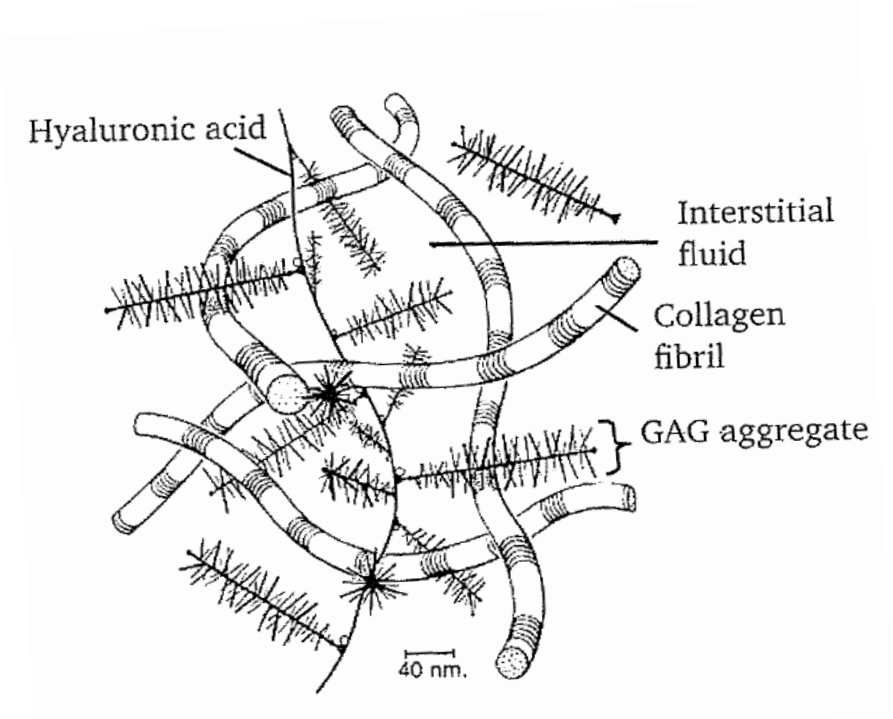

Figure 1.3. The extracellular matrix (ECM) of articular cartilage, including collagen fibres, proteoglycans (PG) and interstitial fluid. The PG protein matrix consists of repetitive chondroitin sulphate and keratin sulphate chains linked to hyaluronic acid (van Mow *et al.*, 1992; van Turnhout *et al.*, 2010). (Reprinted from Biomaterials, Volume 13, van Mow *et al.*, 1992, Cartilage and diarthrodial joints as paradigms for hierarchical materials and structures, pp. 75, Copyright 1992, with permission from Elsevier).

Nutritional impact on articular cartilage

Energy

The role of dietary energy level in AC disorders remains unclear. Some studies in horses and pigs found no effect of *ad libitum* versus restricted feeding management or energy level on OC (Donabédian *et al.,* 2006; Jorgensen *et al.,* 1995). Other studies showed a lower severity of joint lesions at different feeding levels (Goedegebuure *et al.,* 1980; Savage *et al.,* 1993), but a study in gilts showed a detrimental effect of dietary restriction and flushing on cartilage soundness (Slevin *et al.,* 2001). These inconsistent research results can result from environmental and experimental differences, and/or the unconfirmed pathogenic mechanism of strong postprandial hyperinsulinemia (van Weeren, 2006).

Carbohydrates

Research in horses shows that carbohydrates can play a role in AC health (van Weeren, 2006), but these results have not yet been confirmed in sows. The response of high dietary glucose concentrations, and insulin responses to easily fermentable carbohydrates, causes a strong postprandial hyperinsulinemia in horses. This mechanism results in increased insulin and IGF-1 and 2, which have a direct effect on endochondral ossification. Insulin also stimulates removal of the thyroid hormone from the bloodstream, which involves chondrocyte differentiation and blood vessel invasion in growth cartilage (van Weeren, 2006). This may affect the optimal diffuse nutrient supply and supports the idea that high dietary glucose concentrations and insulin responses are predisposing factors for OC in horses (van Weeren, 2006).

The PG-component glucosamine sulphate may stimulate PG synthesis and may therefore be important for AC (Crenshaw, 2006; Percival, 1997). Glucosamine sulphate may be beneficial for symptom alleviation in DJD disorders rather than for OC (Frantz, 2006). For DJD symptom alleviation, it is thought that glycosaminoglycan (GAG) has the ability to inhibit the production of inflammatory mediators that send signals for cartilage degradation, thereby mimicking the effect of omega-3 fatty acids and antioxidants (Percival, 1997). It is also suggested that glucosamine sulphate normalises cartilage metabolism and inhibits cartilage degradation via blocking of matrix metalloproteinase (MMP) production (Frantz, 2006; Percival, 1997).

Proteins and amino acids

The dietary protein concentration as well as the protein source used in sow diets affects the occurrence of OC. The low protein diets fed to gilts may weaken cartilage. This results in an increase in the load borne on the distal caudal lateral bone surface (Slevin *et al.,* 2001).

Supplementing proline and glycine may have a positive effect on collagen formation in sows (Frantz, 2006). However, providing arginine (which can be converted to proline) and glycine showed a higher overall OC severity (abnormality x severity) score and higher number of abnormalities (Frantz, 2006). This may be caused by an excessive conversion of arginine in nitric oxide (NO). This increases signalling inflammatory pathways, resulting in collagen breakdown or chondrocyte apoptosis (Frantz, 2006). Feeding methionine in combination with Mn, threonine, proline and glycine reduces the severity of OC. Methionine may have an important role as S-adenosylmethionine (SAMe) precursor or as an S donor (Frantz, 2006), because, the methionine metabolite SAMe increases the collagen and proteoglycan synthesis (Frantz, 2006).

Lipids

Omega-3 fatty acids (DHA, EPA, and ALA) do not affect OC, but they do have a beneficial effect for symptom alleviation of degenerative AC disorders such as DJD. It does not affect OC, because the AC of animals supplemented with fish oil requires more energy to compress and is therefore less able to distribute mechanical forces and resist tearing (Frantz, 2006). The beneficial effect for DJD symptom alleviation is caused by reduced inflammatory responses or blocked matrix metalloproteinase (MMP) production, which is present in high concentrations during cartilage degradation (Darlington and Stone, 2001; Frantz, 2006).

Minerals

Macrominerals

The specific role for macrominerals in AC metabolism is not clear. Calcium and P are directly involved in the maturation and differentiation of cartilage cells (Crenshaw, 2006) but research in pigs indicates that Ca and P do not affect the severity of OC (Fukawa and Kusuhara, 2001; Nakano *et al.*, 1987; Ytherus *et al.*, 2007). Increasing the dietary electrolyte balance (dEB) reduced leg weakness in pigs but OC lesions were not affected in a study of van der Wal *et al.* (1986). These results were contradicted in another study, in which leg weakness was not improved (Ernst *et al.*, 1990). Supplementation with a wide range of Mg levels did also not affect the incidence and severity of OC lesions (Nakano *et al.*, 1987).

One macromineral, which may have an effect on AC disorders, is sulphur (S), because S is required for proteoglycan (PG) chain formation that extends from hyaluronic acid. It provides the absorptive capacity of cartilage (Frantz, 2006) and chondroitin sulphate necessitates S (McDowell, 2003). However, the effect of S supplementation on OC and DJD has not yet been confirmed for sows.

Microminerals

Zinc supplementation may increase the occurrence of AC related disorders, because Zn exacerbates the incidence of OC in pigs and horses (Frantz, 2006; Semevolos and Nixon, 2007). In the presence of excessive dietary Zn intake, Cu is displaced from metallothionein (MT) and the Cu-dependent enzyme lysyl oxidase is reduced (Hill *et al.,* 1983a; van Weeren, 2006). This mineral imbalance between Zn and Cu caused lameness and abnormal AC of the distal and humerus long bones (*e.g.* thinned AC layer, patches of underlying epiphyseal bone, fractured AC surfaces, cartilage proliferation and sometimes excessive synovial fluid) in gilts and sows fed 5,000 mg/kg ZnO (Hill *et al.,* 1983a).

Copper is required for connective tissue development, because the activity of the key enzyme lysyl oxidase, which is essential for collagen cross-linkage, is Cu-dependent (Frantz, 2006; Percival, 1997). During Cu deficiency, the activity of lysyl oxidase decrease and the collagen will not cross-link and mature (Rucker *et al.,* 1998; McDowell, 2003). This results in reduced leg joint rigidity, excessively flexed hocks, and crooked forelegs. Long-term marginal or inadequate Cu status creates even degenerative and inflammatory conditions, including arthritis and osteoporosis (McDowell, 2003). Copper supplementation to sows may reduce the OC severity of their offspring (Frantz, 2006). However, this positive effect for Cu supplementation is not supported in all studies (Nakano *et al.,* 1987; Ytherus *et al.,* 2007).

Manganese may influence OC in sows as it affects AC by activating galactotransferase and glycosyltransferase. These enzymes play a role in the synthesis of chondroitin-sulphate chains of proteoglycan (PG), which are essential for the normal formation of cartilage (Andrieu, 2008; Tomlinson *et al.,* 2004). However, some studies in pigs indicate that supplementation of a wide range of Mn levels did not affect the incidence and severity of OC lesions (Grøndalen, 1977), while one study showed lower severity of OC scores in sows (Frantz, 2006). In the same study (Frantz, 2006), addition of Mn combined with methionine, proline and glycine numerically reduced the overall severity score of OC.

Low dietary selenium (Se) as well as high dietary molybdenum (Mo) concentrations may negatively influence the occurrence of OC in horses (Semevolos and Nixon, 2007), but this influence is not investigated for sows. In rats, low dietary Se intake resulted in a higher rate of apoptotic chondrocytes in AC (Guo *et al.,* 2015) and high dietary Mo intake in animals interfered with Cu absorption (Palmer, 1993).

Iron (Fe) may have an indirect role in AC metabolism, because Fe is an essential component of the antioxidant catalase in the synovial fluid and Fe is a catalyst for lipid peroxidation and radical formation (Andrieu, 2008; Percival, 1997). Furthermore, Fe is an antagonist of Zn and Cu (Andrieu,

2008; Percival, 1997). For these reasons, dietary Fe concentration should be maintained at adequate levels, but scientific evidence on sows is lacking.

Silicate supplementation may lower AC scores in pigs (Frantz, 2006). In one study with pigs fed Zeolite A supplements, the supplemented pigs tend to have lower AC and overall severity scores compared to pigs fed either a control diet or a diet with leucine, isoleucine and valine added to it (Frantz, 2006). Silicon is mainly found in the proteoglycan (PG) matrix to ensure stability (Frantz, 2006), but silicates, such as Zeolite A, also maximise the prolyl hydroxylase activity which is required for the hydroxyproline synthesis and is thus important for collagen formation (Frantz, 2006; Goff, 2010).

Vitamins

Vitamins, especially vitamin A, B, and D, have not been shown to affect OC (Nakano *et al.,* 1987; Ytherus *et al.,* 2007). Vitamin C may affect AC by regenerating other antioxidants, such as vitamin E. Vitamin C deficiency results in poor collagen synthesis (Percival, 1997), but supplementation fails to prevent or decrease AC disorders and does not influence hydroxyproline concentrations (Nakano *et al.,* 1987; Ytherus *et al.,* 2007). This may be due to the capacity of pigs to synthesise vitamin C from glucose to meet their requirements (Frantz, 2006). Vitamin E may not be effective against OC in pigs. It benefits connective tissue repair (Percival, 1997), but supplementation did not reduce OC (Grøndalen, 1974).

Claw quality and horn production

Anatomy of the claw

The claw (ungulae) of pigs is usually unpigmented and composed of modified skin with a cornified epidermis (Schummer *et al.,* 1981; Mülling and Budras, 2003a). It surrounds the skeletal and soft structures of the distal part of each digit (Figure 1.4). The dewclaws are in principle composed of the same modified skin layers (Schummer *et al.,* 1981). The hairless skin covering the distal part of the digit consists of three layers, the subcutis, dermis and epidermis that are modified in different parts of the hoof to form five segments (e.g. periople, corona, wall, sole and bulb) (Schummer *et al.,* 1981; Mülling and Budras, 2003a). The subcutis is an immovable cushion that consists of a three-dimensional network of connective tissue fibres with enclosed fat lobules (Mülling and Budras, 2003a). The cushion is thick in the bulb (*i.e.* heel) for shock absorption. The dermis consists of a reticular layer and superficial papillary layer. This papillary layer, except the layer in the wall segment, bears dermal papillae. The wall segment presents dermal lamellae. The deep layers of the epidermis correspond to the dermal papillae and lamellae, producing tubular horn in all segments

except the wall and lamellar horn in the wall segment (Schummer *et al.*, 1981; Mülling and Budras, 2003a,b).

The claw segments

The periople segment is closest to the haired skin and the coronary and wall segments follow distally. Horn is formed in these three segments and moves from proximal to distal to form the horny wall (Lamina or Paries corneus). The horn of the sole and bulb segments forms the ground surface of the claw (Figure 1.4) (Schummer *et al.*, 1981; Mülling and Budras, 2003a).

Periople segment (Limbus). The periople dermis covers the subcutis and bears distally directed perioplic papillae. The perioplic epidermis covers the dermis and forms horn tubules on the dermal papillae. This soft horn represents the external layer of the wall segment. (Schummer *et al.*, 1981; Mülling and Budras, 2003a).

Coronary segment (Corona). This segment is located distally from the periople segment and extends halfway down the claw. The coronary dermis covers the subcutis and bears conical coronary papillae. The coronary epidermis forms horn tubules corresponding to the dermal papillae. This horn forms the middle layer of the wall segment. (Schummer *et al.*, 1981; Mülling and Budras, 2003a).

Wall segment (Paries). The wall segment is located distally from the coronary segment and does not have a subcutis. The lamellar (parietal) dermis bears proximodistally oriented dermal lamellae. No secondary lamellae are present in pigs and cattle compared with horses. The (Parietal) epidermis of the wall bears epidermial lamellae between the dermal lamellae. These epidermal lamellae are cornified in the middle layer to form horny lamella). (Schummer *et al.*, 1981; Mülling and Budras, 2003a).

Sole segment (Solea). The sole segment includes the anterior part of the ground surface and does not have a subcutis. The solear dermis bears transverse ridges topped by dermal papillae. The papillae are arranged in rows. The solear epidermis contains the horn tubules. (Schummer *et al.*, 1981; Mülling and Budras, 2003a).

Bulbar segment (Torus ungulae). The bulbar segment has a marked distal bulge and encompasses the posterior half of the ground surface. The subcutis of the bulbar segment forms the digital cushion and consists of connective and adipose tissue. This digital cushion is covered by the bulbar dermis and the deep flexor tendon or third phalanx. The bulbar dermis bears dermal papillae, which arise partly from wavelike ridges. The bulbar epidermis covers the dermis containing horn tubules. (Schummer *et al.*, 1981; Mülling and Budras, 2003a).

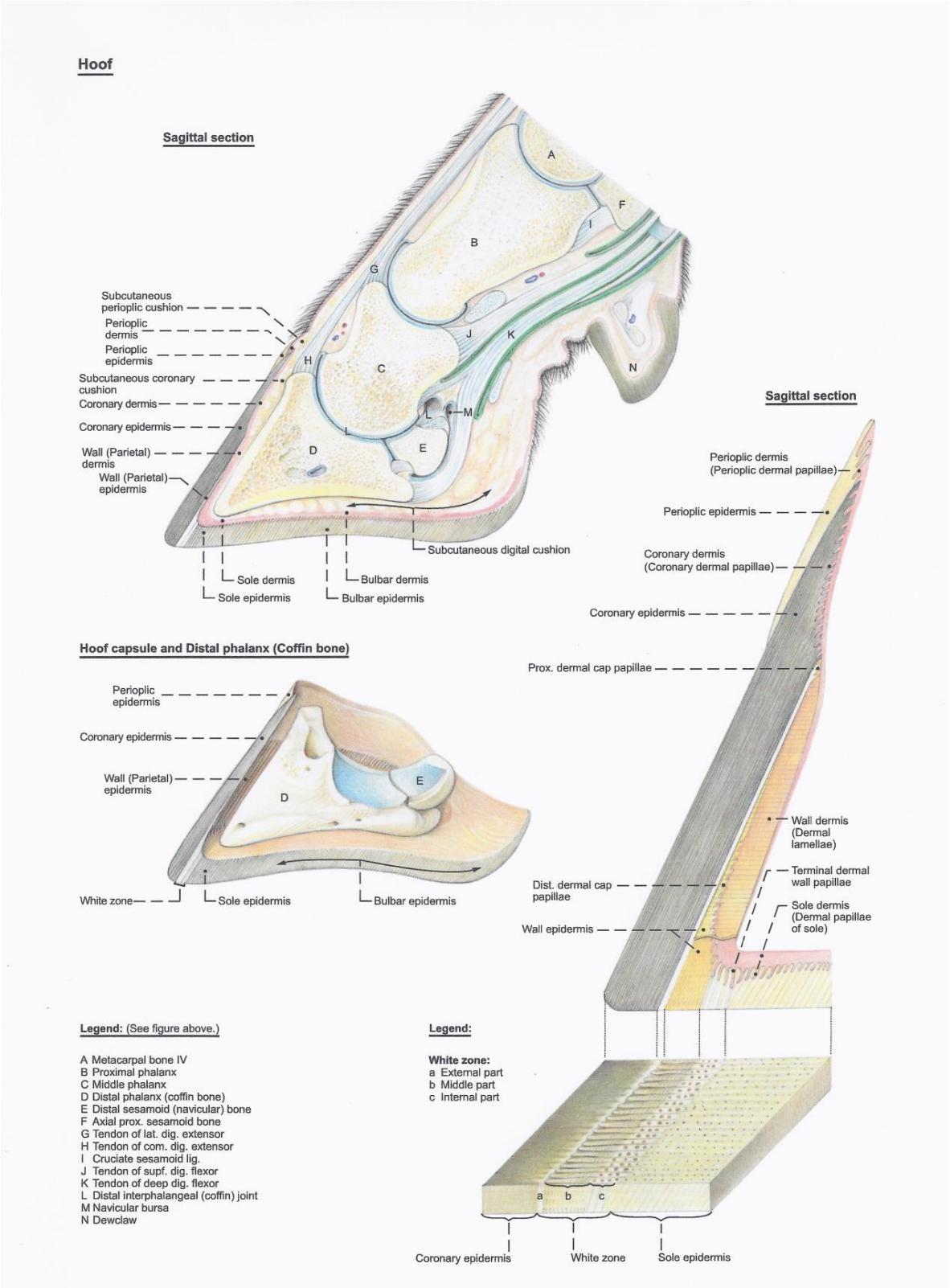

Figure 1.4. Anatomy of the claw, represented by a sagittal section of the bovine claw. The claw of pigs and ruminants are generally similar, except for the dewclaw which contains a similar osseous skeleton of the main digits in pigs (Adapted and reprinted with permission of Budras *et al.*, 2003, Bovine Anatomy, 1st Edition, Schlütersche GmbH & Co. KG, Hannover, Germany).

The claw capsule

The cornified claw capsule consists of the horny wall that includes the three layers formed by the perioplic, coronary and wall segments and the epidermis of the sole and heel (Mülling and Budras, 2003b; Ossent, 2010) (Figure 1.4). The external, middle and internal layers are bonded together, in which the external layer is thin, the middle layer represent the bulk of the lamina and the internal layer bears the horny lamellae that is part of the junctional horn (Mülling and Budras, 2003b). The junctional horn includes all tissues that attach the distal phalanx to the inside of the lamina. The height of the wall gradually decreases from the toe to the heel (Schummer *et al.*, 1981).

Distally on the wall-sole border, the almost vertically oriented dermal lamellae bend into horizontal directed dermal ridges of the sole segment. At the bend, the lamellae are split into terminal dermal papillae (Schummer *et al.*, 1981; Mülling and Budras, 2003b). These are covered with the epidermis forming terminal tubular horn. In this part of the white zone, the terminal horn fills the spaces between the horny lamellae. The white zone (*i.e.* white line) consists only of horn produced by the wall segment and includes an external, middle and internal part (Mülling and Budras, 2003b). The white zone forms the connection between the bearing surface of the wall and the horny sole (Schummer *et al.*, 1981).

Histological structure of the claw

A horizontal section of the claw shows the dermis covered by densely arranged cells of the epidermal stratum basale (Figure 1.5) (Tomlinson *et al.*, 2004; Cameron, 2012). Nutrients and hormones are provided from the dermis to the stratum basale for the continuously production of epidermal cells. The distal layer adjacent to the stratum basale is the stratum spinosum. The stratum spinosum forms the border between differentiation and cornification and basophilic dense keratohyalin granules accumulate in the cells (Tomlinson *et al.*, 2004; Cameron, 2012). Epidermal cells turn into horn cells establishing the stratum corneum (Tomlinson *et al.*, 2004; Cameron, 2012).

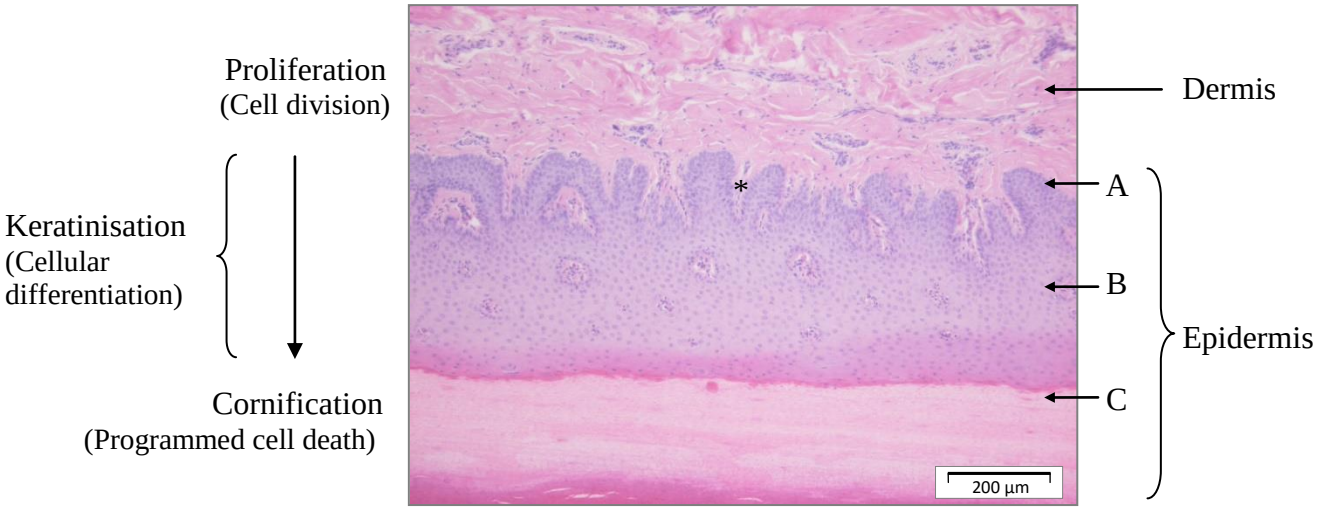

Figure 1.5. Horizontal section of the horn wall of sows (10x magnification of the objective, HxE stain). The dermis including the dermal lamellae (*) is covered by the densely arranged cells of the stratum basale of the epidermis (A). At the stratum basale, proliferation starts. The distal layer adjacent to the stratum basale is the stratum spinosum (B) were keratinisation occurs and keratin proteins are formed. Synthesis of the ICS occurs more distally in the stratum spinosum. Cornification is the final step of the keratinisation process. Keratinocytes cornify and transform into horn cells by intermolecular cross linkages with ICS forming the stratum corneum (C) (Tomlinson *et al.,* 2004; Cameron, 2012).

Claw lesions and horn production

The strength of the claw varies between the soft and hard tissue claw areas, and the junction between these areas is particularly susceptible to lesions (Anil *et al.,* 2007; Ossent, 2010). This may be a result of a different mineral composition. Calcium, P, Cu, and Zn levels are higher in the harder keratins of the wall, while the softer keratins in the heel contain more water, Na, K, and Fe (Anil *et al.,* 2007; Van Amstel *et al.,* 2009).

Claw lesions include heel overgrowth and erosions, separations and cracks along the heel/sole junction, separations and cracks along the white line, horizontal and vertical wall cracks, skin lesions near the claw, and excessive (dew) claw length (Figure 1.6) (Anil *et al.,* 2005). The presence of claw lesions, mainly white line and vertical wall cracks, causes lameness in 5 to 20% of all cases (Anil *et al.,* 2007).

Figure 1.6. The various types of claw lesions in sows. A: haemorraghes, B: severe heel erosion, C: horizontal wall crack, D: overgrown dewclaw, heel erosion, and separation of the heel/sole junction.

The primary causes for claw lesions in sows are related to the horn quality: trauma and mechanical factors along with inferior horn including excessive or inadequate wear (Ossent, 2010; Torrison, 2010). Claw lesions that are almost invariably a result of inflammation (*e.g.* laminitis, abcess and necrosis and ulcers) will not be further discussed within this thesis (Ossent, 2010).

The integrity of the claw and thus the susceptibility to claw lesions depends largely on the quality of horn production (Torrison, 2010). Claw horn production is the result of proliferation, keratinisation (cellular differentiation) and cornification (cell death) of keratinising epidermal cells (keratinocytes) in the claw epidermis (Figure 1.5) (Tomlinson *et al.*, 2004). **Keratinisation** represents the synthesis, aggregation and stabilisation of keratin proteins by keratinocytes. The keratin proteins are cross-linked by the formation of disulphide (hard horn) or sulfhydryl (soft horn) bonds, which consist of a stable protein complex. Keratinisation also includes the synthesis and extrusion of intercellular cementing substance (ICS) into the intracellular space (Muelling, 2009; Tomlinson *et al.*, 2004). The ICS consists of glycoproteins and complex lipids, such as phospholipids, glycolipids and acylglycosylceramides. They establish cell to cell adhesion with the keratin proteins during cornification. **Cornification** is the final step of the keratinisation process, where the keratohyalin granules are dissolved and merged to filamentous keratin proteins. The keratinocytes cornify and transform into horn cells (*i.e.* die) by intermolecular cross linkages with ICS (Tomlinson *et al.*, 2004). The keratin proteins and ICS provide chemical and mechanical stability to the horn. Especially the ICS lipids provide an intracellular barrier to prevent excessive loss of water as well as extreme hydration (Muelling, 2009; Pollitt, 2004; Tomlinson *et al.*, 2004). The sulfhydryl groups in the periople, heel horn and white line provides elasticity (Pollitt, 2004).

Horn production depends on nutrition, particularly the diffuse nutrient, oxygen and hormone supply from the blood via the dermis to the avascular epidermis. The dermis controls and modulates this

nutrient supply to the epidermis (Muelling, 2009; Tomlinson *et al.,* 2004). When the nutrient supply of amino acids, fatty acids (linoleic and arachidonic acid), minerals and vitamins is insufficient, the horn production will be disturbed with an increased susceptibility to chemical, physical or microbial damage from the environment (Muelling, 2009; Tomlinson *et al.,* 2004). In particular, nutrient deficiencies that lead to inferior ICS production or predispose ICS to excessive oxidative damage may cause inferior horn production and increase the risk of claw lesions, such as cracks and wear (Tomlinson *et al.,* 2004). The diffuse supply is impaired during mechanical overload and tissue compression, especially in case of perfusion and damage of the vascular walls (Muelling, 2009; Vermunt and Greenough, 1995). Factors associated with perfusion and damage of the vascular walls are metabolic activity (*e.g.* metabolic stress during parturition and lactation), systematic diseases, histamine, lactate, endotoxin, activation of matrix metalloproteinase (MMPs), and vasoactive factors (*e.g.* serotonin and bradykinine) (Muelling, 2009; Pollitt, 2004).

Nutritional impact on claw quality and integrity

Proteins and amino acids

Horn production may be impaired during late gestation and lactation, because the amino acid requirements of sows are high during these periods (NRC, 2012) and the sow may not be able to produce enough proteins to meet the demands, especially when dry matter intake is decreased during early lactation. This shortage of protein during early lactation may result in insufficient protein synthesis by the developing keratinocytes, which may interfere with horn production and result in an increased risk for lameness as has been reported for cattle (Tomlinson *et al.,* 2004). Sulphur-containing amino acids are important during keratinisation, an important contributor to claw integrity. Cysteine is especially relevant, because the disulphide bonds between cysteine-residues are an integral part during the final step of keratinisation and cornification. Cysteine is preferred for partially keratinised epidermal laminea and establishes the cellular envelope, which provides high cell-wall resistance and rigidity against proteolytic enzymes (Tomlinson *et al.,* 2004). The effect of methionine supplementation on claw quality is not known for sows. In cows, supplementation of methionine results in softer horn, because it limits the cysteine concentration, which helps to form disulphide bridges (Vermunt and Greenough, 1995). However, another study in heifers reported no significant differences in horn hardness despite different methionine and melatonin concentrations in the diet (Galbraith *et al.,* 2006).

Lipids

Fatty acids may have a beneficial effect on the claw resistance to environmental challenges and the levels of fatty acids are easily influenced by the diet (Wood *et al.,* 2008). In horses, supplementation of feed with a primrose oil mixture resulted in significant differences in the perioplic lipid fraction of the hoof wall, with increased cholesterol esters and partial glycerides and a reduction in free cholesterol (Reilly *et al.,* 1998). However, this study did not investigate the consequence of the changed lipid fraction on horn production. For sows, research is warranted.

Minerals

Macrominerals

Calcium influences horn production through the initiation and regulation of keratinisation and cornification. This occurs via the activation of epidermal transglutaminase (TG). Epidermal transglutaminase cross-links the keratin fibres on the cell wall via glutamyl-lysine bonds to form a ridged cell wall (Mülling *et al.,* 1999; Tomlinson *et al.,* 2004). Insufficient dietary Ca intake reduces the amount of available Ca and lowers plasma Ca concentrations, thereby interfering with the Ca quantity provided to the keratinocytes. This disturbs the diffuse vascular supply. As a results, TG activity decreases and dyskeratotic horn is produced (Mülling *et al.,* 1999; Tomlinson *et al.,* 2004). Elevated dietary Ca as well as P concentrations have no effect on the incidence and severity of toe lesions and overall structural sow soundness during three parities, but it seems that hind claws respond more to elevated Ca and P levels compared to front claws (Arthur *et al.,* 1983).

Microminerals

Microminerals are very important for horn production, not only in terms of dietary intake but also in term of bioavailability. Increasing the bioavailability may improve the absorption in the lumen of the small intestine and therefore improve claw integrity (Andrieu, 2008; Kessler *et al.,* 2003; Tomlinson *et al.,* 2004). Microminerals can be added to the diet as complexed organic microminerals, but their bioavailability is increased even more when these complexed organic microminerals are combined together in the diet (Anil *et al.,* 2010a; Bradley, 2010). A sufficient supply of bioavailable microminerals is very important, and the demands increase during late gestation and early lactation (McDowell, 2003; NRC, 2012). Insufficient dietary micromineral availability or an interrupted diffuse supply to the keratinocytes thus results in inferior horn integrity (Ballantine *et al.,* 2002; Tomlinson *et al.,* 2004) and may increase the risk for sow lameness.

Zinc is an important micromineral that influences horn production. The catalytic, structural, and regulatory Zn functions all influence the processes required for horn production. The **catalytic** Zn function includes the activation of Zn-dependent metalloenzymes (*e.g.* alkaline phosphatase (ALP), carbonic anhydrase, alcohol dehydrogenase, RNA polymerase, and RNA nucleotide transferase). These metalloenzymes increase the cellular activity during keratinocyte differentiation (Hendry *et al.,* 1997; Tomlinson *et al.,* 2004), thereby supporting the keratinisation of keratin proteins. The **structural** function of Zn is related to the role of Zn finger proteins in protein-to-protein interactions, which are thought to affect cellular proliferation and differentiation (Tomlinson *et al.,* 2004). Low Zn status may result in inhibited Zn finger protein formation that will disturb the formation of keratin filaments needed in the maturing keratinocyte. This may interfere with the formation of disulphide bonds, necessary for horn production. The **regulatory** function of Zn is to regulate protein kinase C, which is Ca-dependent and stimulates the phosphorylation of proteins to provide energy. This energy is used in the differentiation process of keratinocytes and is thus important for horn production. Zinc also regulates calmodulin, inositol phosphate synthesis, thyroid hormone binding, and copper/zinc superoxide dismutase (Cu/Zn SOD). Calmodulin is important for Ca binding as well as for the transport of Ca into the cell cytosol. Inositol phosphate mobilises Ca from intracellular stores, and thyroid hormones regulate these calmodulin and protein kinase C actions (Andrieu, 2008; Kellon, 2008; Tomlinson *et al.,* 2004). Copper/zinc superoxide dismutase prevents lipid peroxidation and is relevant for the lipids present in the intercellular cementing substance (ICS). Sustaining ICS is essential for the structural integrity and function of the claw (Mülling *et al.,* 1999). Disturbance of Cu/Zn SOD may result in higher fragility of the cell membranes due to the unsaturated lipids in the cell periphery. These are particularly vulnerable to oxidative damage (Tomlinson *et al.,* 2004). In addition, the Zn-dependent matrix metalloproteinase (MMPs) (especially MMP2 and MMP9) is activated during keratinocyte remodelling, whereas MMP's degrade ECM and the basement membrane (Pollitt, 2004).

For copper, claw integrity is more affected by insufficient dietary Cu availability rather than toxicity. Claw lesions, such as cracks and abscesses, may occur as a result of an insufficient dietary Cu availability (Tomlinson *et al.,* 2004). Insufficient dietary Cu may reduce the cytochrome oxidase activity, which will reduce respiration and oxidative phosphorylation, thereby decreasing the energy supply needed for differentiating keratinocytes (Tomlinson *et al.,* 2004). Copper deficiency also reduces Cu/Zn SOD activity, which may affect claw integrity as described above under Zn (Tomlinson *et al.,* 2004). One important activity of Cu is activation of lysyl and thiol oxidases and ceruloplasmin. Intracellular thiol oxidase is essential for the disulphide bond formation between cysteine residues of keratin filaments and provides structural strength and rigidity to the keratinised

cell matrix (Kellon, 2008; Tomlinson *et al.*, 2004). Although Cu toxicity is uncommon in sows (McDowell, 2003), excessive accumulation of Cu results in oxidative stress, which affects the ICS (McDowell, 2003).

Manganese is important for the activation of the enzyme pyruvate carboxylase, which is essential for horn production. Manganese influences gluconeogenesis and cellular energy production. It also activates Mn SOD, which removes free radicals. As a result, the lipids of the ICS are protected against lipid peroxidation (Andrieu, 2008; Tomlinson *et al.*, 2004). If the animal is deficient in Mn, Mg can partially substitute Mn (McDowell, 2003).

The main role of Se, which is closely linked to vitamin E, is to protect the intra- and extracellular lipid membranes against oxidative damage and thus protect the ICS (Muelling, 2009; Tomlinson *et al.*, 2004). This protective function is ensured by the selenoprotein glutathione peroxidase, because it converts hydrogen peroxidase and free oxygen to water. Selenium is also incorporated in the selenoenzyme called deiodinase. This enzyme catalyses the inactive thyroid hormone to the biologically active form, triiodothryonine (McDowell, 2003). The relation of thyroid hormones to horn production is described above under Zn. When dietary Se concentrations are too low, the anti-oxidant function is compromised in cattle (Andrieu, 2008), whereas supplementation results in an inhibited or reduced amount of disulphide bonds formed during cornification. The cysteine and methionine part of keratin fibres are then replaced by Se into selenocysteine (SeCys) or selenomethionine (SeMet). This replacement creates inferior horn with poor rigidity (Muelling, 2009). In Se toxicity, the symptoms of inferior horn are aggravated, resulting in lameness and hoof malformation (McDowell, 2003).

Chromium (Cr) is an essential micromineral, because it is a cofactor in insulin activation by forming a complex between insulin and insulin receptors. Insulin increases glucose uptake and thereby maintains energy production and cell metabolism (Appelt, 2006). If the glucose uptake is affected, the glucose metabolism will be impaired, causing a disturbed diffuse nutrient supply from the dermis to the epidermis. A disturbed nutrient supply relates to inferior horn production, which may result in claw lesions and lameness. The cell glucose metabolism can be improved by Cr and the vitamin niacin, because they are responsible for the formation of glucose tolerance factor (GTF). This GTF is ineffective without Cr in animals (McDowell, 2003) and when GTF is ineffective, the diffuse nutrient supply may be interfered with.

Dietary iodine (I) may be important for optimal horn production mainly in young pigs. In older sows, I deficiency is uncommon (McDowell, 2003). Iodine is essential in thyroid hormone synthesis, thyroxine and triiodothyronine. As mentioned above under Zn, these hormones regulate

the calmodulin and protein kinase C action necessary for keratinisation and cornification and control the oxidation rate of cells (Tomlinson *et al.*, 2004).

Molybdenum (Mo) is necessary for metabolism of sulphur-containing amino acids (Appelt, 2006). The Mo metalloenzyme, aldehyde oxidase, may be involved in niacin metabolism. Both of these metabolisms affect horn quality and integrity, because sulphur-containing amino acids are important during keratinisation, an important contributor to claw integrity, and niacin involves the formation of GTF, influencing the diffuse nutrient supply to the epidermis (McDowell, 2003; Tomlinson *et al.*, 2004).

Vitamins

Vitamins may have a supportive role in claw integrity. For example, vitamin A is required for cell differentiation. Once bound to receptors that stimulate cell division, it stimulates or inhibits gene expression. In this way, vitamin A deficiency results in poor horn growth and calcification in horses and poultry (Kellon, 2008; Waldenstedt, 2006).

Also, the transition of lysine and proline into hydroxylysine and hydroxyproline during collagen synthesis is necessary for optimal weight bearing and a stable position of the pedal bone in the horn capsule (Muelling, 2009). Vitamin C is important for this transition and therefore the collagen synthesis occurring in the horn of cattle (Muelling, 2009).

Biotin is the vitamin that has received the most scientific attention in relation to pigs' claw health. Biotin is an important water-soluble B vitamin and is required in the keratinisation process to ensure horn integrity. Biotin is also essential for lipid molecule production for the intercellular cementing substance (ICS) complex (Mülling *et al.*, 1999). In pigs, varying results for biotin supplementation have been found. Some studies reported improved claw strength and hardness, increased horn tubules density in the stratum medium, tightly packed keratinocytes and ICS, clearly defined tubules, and a reduced number of claw lesions (Kempson *et al.*, 1989; Webb *et al.*, 1984), whereas no effect of biotin were observed in other studies (Lewis *et al.*, 1991; Penny *et al.*, 1980). These different results may be caused by differences in the amount and duration of supplementation, biotin bioavailability, biotin status at the start of the study, and age and parity of the sow. When supplementing biotin, dietary concentrations higher than 200 ng/kg dry matter (DM) are necessary to heal claw lesions in sows (Misir and Blair, 1986). In terms of age, Simmins and Brooks (1988) observed significantly fewer claw lesions in the biotin-supplemented group compared to the control group from d170 to first weaning. However, no differences were found before 170 days of age. Further research is required.

Vitamin E (lipid-soluble cellular antioxidant, works synergistically with Se) plays an important role for the lipid-rich ICS and thus supports claw integrity, because it prevents tissue breakdown and membrane degeneration from oxidative degeneration (Mülling *et al.*, 1999; Tomlinson *et al.*, 2004; Waldenstedt, 2006). Insufficient vitamin E levels in cattle increase this lipid peroxidation of cellular membranes, resulting in decreased energy production and increased occurrence of lesions (Tomlinson *et al.*, 2004).

Zinc: the essential micromineral

As mentioned above, Zn is important for the keratinisation process. As an essential micromineral, its metabolism is tightly regulated to maintain homeostasis. Homeostasis reflects the equilibrium in the flow of a nutrient within an organism (Kirchgessner, 1993). The homeostasis of Zn is controlled by the epithelial cells of the small intestine, liver and pancreas (Buckley and D'Mello, 2000; Krebs, 2000; Peters, 2006) and is maintained through the regulation of absorption and (endogenous) excretion (Buckley and D'Mello, 2000; King *et al.*, 2000; Hill and Link, 2009). For example, if the dietary Zn intake decreases, the efficiency of Zn utilisation increases, resulting in a higher efficiency during absorption and a decreased (endogenous) Zn excretion (King *et al.*, 2000).

Zinc metabolism

Absorption

Between 5 and 60% of dietary Zn is absorbed in the small intestine (Pond *et al.*, 1995; Buckley and D'Mello, 2000; McDowell, 2003). In the small intestine, it crosses the brush border of the lumen (apical site) into the enterocyte (basolateral site) (King, 2000; Krebs, 2000; Peters, 2006). Within the enterocytes, the transfer of Zn is facilitated and regulated by saturation of the metal binding protein metallothionein (MT) (McDowell, 2003; Cousins *et al.*, 2006; Peters, 2006).

Distribution

After absorption, Zn in the plasma is incorporated into proteins and transported predominantly loosely bound to albumin and to a lesser extent to α2-macroglobulin and high molecular weight binding ligands (Underwoord and Suttle, 1999; Buckley and D'Mello, 2000; McDowell 2003). As a result of increased plasma Zn concentrations, MT is synthesised in the liver (hepatic MT) for removing Zn from the plasma and dividing it between various tissues (Pond *et al.*, 1995; Underwood and Suttle, 1999; McDowell, 2003). All of the absorbed Zn passes the plasma rapidly, before it reaches the different tissues, to maintain a constant concentration of plasma Zn (King,

2000). The distribution of Zn from the blood to other tissues results in saturation of one tissue and translocation of Zn to unsaturated tissues (Pond *et al.,* 1995).

Storage and redistribution

Zinc is found in all body tissues, although liver and bone are the major storage tissues. The storage capacity of Zn that can be rapidly mobilised is limited. Only a few readily available Zn stores in the body are present, the so-called exchangeable Zn pool (King, 1990 and 2011; Buckley and D'Mello, 2000; McDowell, 2003). This pool is partly located in plasma, liver and bone tissue (King, 1990 and 2011). Reduced Zn intake cannot exert changes in Zn concentration in hair, skin, heart and skeletal muscle (these concentrations remain constant), whereas plasma, liver, bone, and testes Zn increases or decreases dependent of dietary Zn intake (King, 2000; McDowell, 2003). To maintain Zn in tissues, redistribution of Zn between tissues appears to be an important mechanism coordinated by Zn transporters and MT (Osredkar and Sustar, 2011).

Excretion

Zinc is mainly excreted via the faeces and to a lesser extent via urine (Krebs, 2000; McDowell, 2003; Peters, 2006). The faecal Zn excretion consists of two constituents: 1) the unabsorbed Zn fraction and 2) the endogenous loss in excess of the unabsorbed Zn fraction that contributes to Zn homeostasis by increasing or decreasing the retention of absorbed Zn (Weigand and Kirchgessner 1980; Pond *et al.,* 1995). Other losses occur through losses of keratinised surfaces (King, 2000).

Functions of zinc

Zinc is required for multiple metabolic functions and is thus essential for normal metabolism, whereas other nutrients are required for specific metabolic functions, such as Fe and Se (King, 2011). Therefore, Zn is omnipresent and has important catalytic, structural, and regulatory functions (McDowell, 2003; King, 2011; Naithani *et al.*, 2014).

Many enzymes depend on Zn for their catalytic activity (King, 2011; Naithani *et al.,* 2014), such as the metalloenzymes alkaline phosphatase (ALP) and carbonic anhydrase (Underwood and Suttle, 1999; Suttle, 2010). The structural role of Zn facilitates maintenance of enzyme structures and protein folding (King, 2011; Naithani *et al.,* 2014). Proteins involved in cellular differentiation, signal transduction and transcription form a Zn-finger motif, which have cysteine and histidine residues that allow Zn to be bound in a tetrahedral complex of proteins (King, 2011; Naithani *et al.,* 2014). Zinc regulates gene expression; Zn is transported into the cell cytosol or nucleus where it interacts with a metal-binding transcription factor (MTF) and binds to a metal response element

(MRE), stimulating transcription (King, 2011: Naithani *et al.*, 2014). More than 2000 transcription factors that regulate gene expression require Zn (Van paemel *et al.*, 2010).
These biochemical functions of Zn are required for optimal growth and development, sense of taste and smell, wound healing, immune function, skin, hair and nail/claw quality, bone metabolism, hormone regulation, protein metabolism, lipid metabolism, normal brain and central nervous system functioning, antioxidant defence, and for antimicrobial effects (McDowell, 2003; Peters, 2006; Osredkar and Sustar, 2011).

Based on literature, we defined insufficient (below requirements, marginal), adequate (between requirements and maximum allowance), excessive (above maximum allowance until 550 mg Zn/kg) and pharmacological concentrations (above 551 mg Zn/kg, therapeutic dosages). This terminology will be further used throughout the thesis.

The effect of insufficient zinc levels

To facilitate the above functions, sufficient Zn should be absorbed from the diet. However, the amount of Zn absorbed and excreted depends on the dietary Zn concentration and on other dietary as well as animal related factors, such as Zn availability (presence of phytate and phytase), presence and amount of other minerals (Ca, Fe, Cu, Cd), and physiological state of animals (Buckley and D'Mello, 2000; Krebs, 2000; Suttle, 2010).
Insufficient dietary Zn intake in humans and animals results in biochemical and clinical manifestations over time evolving from depletion of body Zn stores to Zn deficiency, dysfunctions and disease (Figure 1.7) (Underwood and Suttle, 1999; McDowell, 2003; Suttle, 2010). The clinical manifestations include reduced appetite and reduced growth and development accompanied by abnormalities in skin and appendages (parakeratosis, Figure 1.8), anorexia, skeletal disorders and inferior claw integrity, impaired immune function, and reproductive disorders. Additional clinical signs are scouring, vomiting, and in severe cases even death (Underwood and Suttle, 1999; McDowell, 2003; Suttle, 2010).

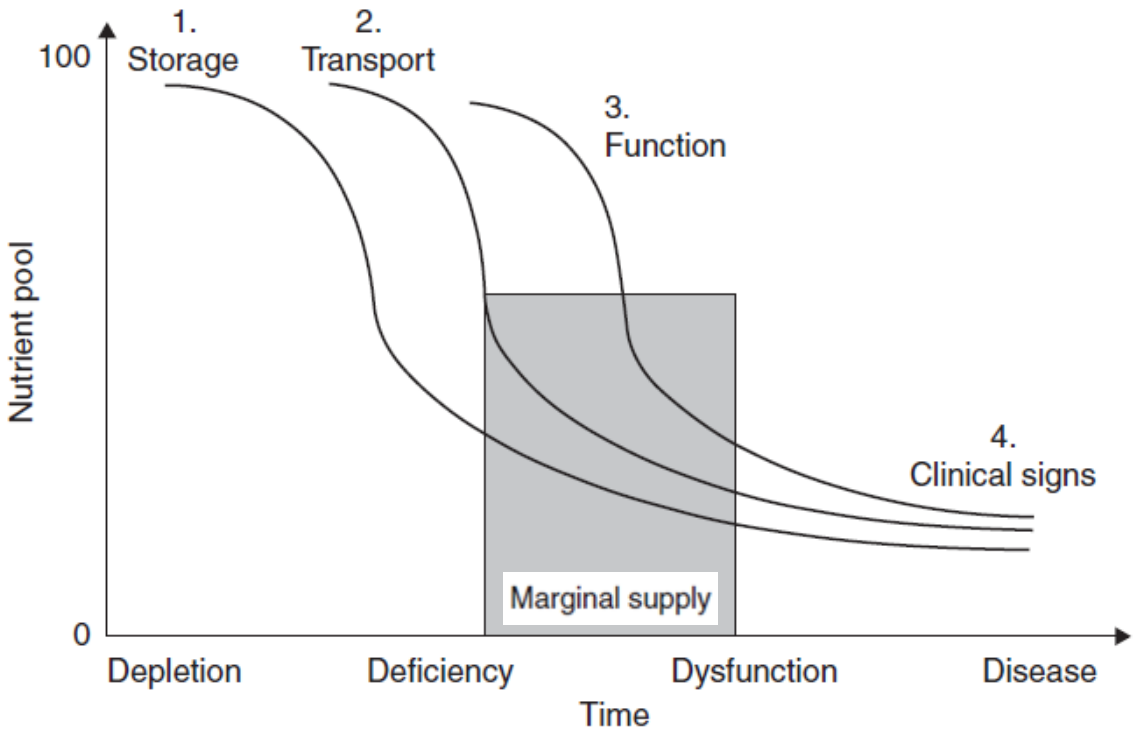

Figure 1.7. Progressive stages of insufficient dietary Zn intake. The phases, depletion, deficiency, dysfunction and disease are related to changes in Zn concentration in body tissues and fluids that serve storage (*e.g.* liver and bone), transport (*e.g.* plasma) or functional (*e.g.* enzymes) purposes. The marginal area (shaded) represents the stage were stores are all but exhausted and Zn-dependent functions begin to fail. The animal remains clinically healthy. The upper limit of 100 on the Zn pool (y-axis) represents the maximum or normal attainable pool size (Adapted and reproduced with permission of Suttle, N.F., 2010, Mineral Nutrition of Livestock, 4th Edition, CAB International, Wallingford, UK).

Zinc responsive parakeratosis and claw lesions

Parakeratosis is a clinical manifestation of insufficient dietary Zn intakes (Figure 1.8) and is reflected by thickening, hardening and fissuring of the skin at the extremities and/ or flanks in pigs (Underwood and Suttle, 1999; McDowell, 2003; Suttle, 2010). The keratinisation of the skin is characterised by the retention of nuclei in the stratum corneum (Lazar and Wang, 2013). This pathogenesis may relate to the pathogenesis of claw lesions, especially for heel horn overgrowth and erosion, because heel horn overgrowth and erosion is also characterised by thickening of the horn (Ossent, 2010). The thickening of the heel horn results in hyperkeratinisation of the epidermis (*i.e.* callus) (Ossent, 2010). This hyperkeratosis reflects hyperplasia of the stratum corneum, which is often associated with an abnormal quantity of keratin (Lazar and Wang, 2013) and differs from the pathogenesis of Zn responsive parakeratosis.

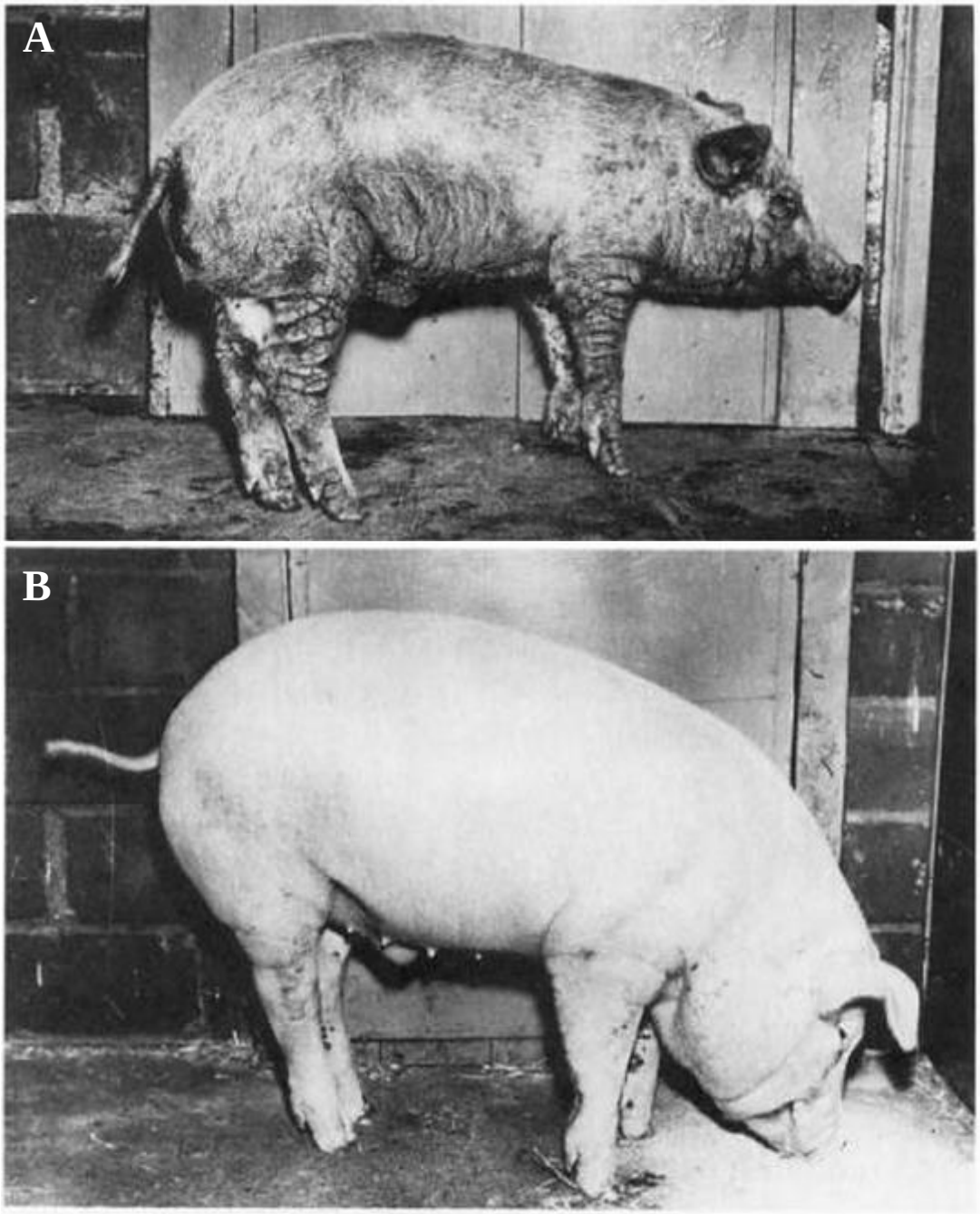

Figure 1.8. Clinical manifestation of insufficient dietary Zn intake. A, pig with parakeratosis. B, same pig 41 days later after addition of Zn to the diet (Reprinted from Swine feeding and nutrition, Cunha, T.J., Mineral requirements of the pig, pp. 64, Copyright Elsevier, 1977) (Courtesy of Hanson, L.E., University of Minnesota, USA).

The effect of excessive zinc levels

Excessive dietary Zn intake in humans and animals results in biochemical and clinical manifestations over time evolving from accretion of body Zn stores to Zn excess, dysfunctions and disease (Figure 1.9) (Underwood and Suttle, 1999; Suttle, 2010). Production animals seem considerably tolerant to excessive dietary Zn concentrations, depending on species and dietary Ca, Cu, Fe, and Cd concentration (Underwood and Suttle, 1999; McDowell, 2003; Suttle, 2010). Non-ruminants have a higher tolerance level than ruminants (McDowell, 2003; Suttle, 2010; Van paemel *et al.*, 2010). Nevertheless, the maximum tolerable level is set at 1000mg Zn/kg (NRC, 2005).

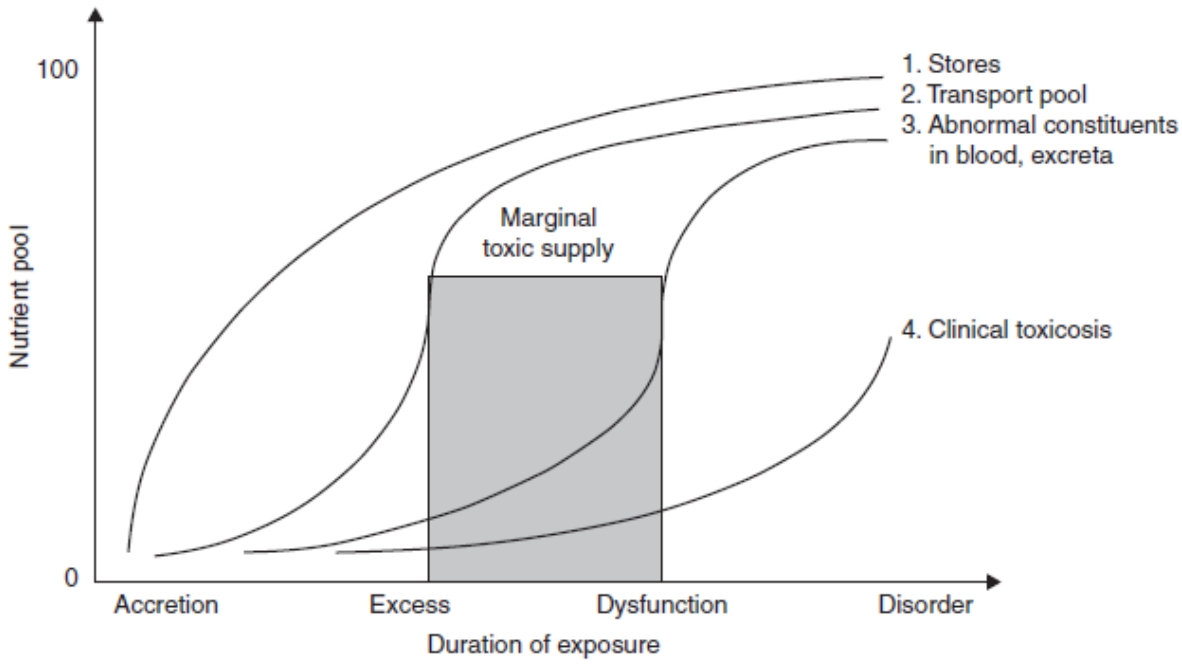

Figure 1.9. Progressive stages of biochemical events during chronic exposure to excessive dietary Zn intake (Adapted and reproduced with permission of Suttle, N.F., 2010, Mineral Nutrition of Livestock, 4th Edition, CAB International, Wallingford, UK).

However, appetite and accompanied feed intake and growth are depressed, vomiting and diarrhoea occur, mortality increases and marginal Cu and Fe deficiency is exacerbated in case of Zn toxicity (McDowell, 2003; Bikker and Jongbloed, 2014). Still, pharmacological dietary Zn intake (2000-3000 mg added Zn/kg diet) may have growth promoting and antimicrobial properties, preventing post-weaning diarrhoea (Hill *et al.*, 2001; Suttle, 2010; Van paemel *et al.*, 2010). These excessive dietary Zn concentrations, including pharmacological Zn concentrations, have a negative impact on the environment.

Toxicity and environment

Zinc is essential for pigs, yet it is also essential for plants, water and soil. Zinc is an abundant element in the earth's crust (between 10 and 300 mg Zn/kg DM) and is found naturally in the air (as fine dust particles that ultimately deposited over land and water), water (dissolved or deposited in the bottom) and soil (not dissolved but bound) (Singh, 2005; Kaur *et al.*, 2014). Pure Zn is a bluish-white shiny metal and amphoteric in nature (Singh, 2005; Kaur *et al.*, 2014). Most rocks and minerals contain Zn and there are approximately 55 mineralised forms of Zn. Zinc has two oxidation states Zn and Zn^{2+} and forms a variety of compounds including Zn oxide or Zn sulphate (Singh, 2005; Kaur *et al.*, 2014).

These Zn compounds are often supplemented to the diet of pigs, because Zn is an essential element in trace amounts (Singh, 2005) and generally the availability of Zn from the ingredients is not sufficient to meet the Zn recommendations for pigs (NRC, 2012). When dietary Zn intake exceeds requirements, a large part of ingested Zn is excreted to the environment. Many of these Zn compounds (*i.e.* salts) are soluble in water and have half-life times being longer than 200 days (Singh, 2005). When concentrations in the environment rises to an extent that the ecosystem is affected, the function and structure (*e.g.* species diversity) of the ecosystem are consequently affected leading to ecotoxicity (Singh, 2005). The amount of Zn becomes toxic for plants and results in excessive Zn accumulation and food chain toxicities (Singh, 2005).

Therefore, the amount of supplemented Zn is limited within the European Union to an authorised maximum total dietary Zn concentration of 150 mg Zn/kg diet to prevent excessive excretion to the environment. In response to this maximum allowance, the use of more bioavailable Zn sources has gained interest.

Still, the maximum allowance probably exceeds the Zn recommendations for pigs (Table 1.1). There is a need to find an adequate balance between requirements and maximum allowances in order to have a sustainable system that is optimal for animal health and animal production, that minimise excretion to the environment, and that provide animal products with an adequate Zn content (López-Alonso, 2012a).

Zinc requirements for pigs

The Zn recommendations for pigs (Table 1.1), especially sows, are based on a limited number of dated studies as reported by Jongbloed *et al.* (2010) and Bikker and Jongbloed (2014). More research is required to determine if the recommendations are still valid for sows. Zinc requirements have been estimated by 1) associations between field disorder and dietary Zn concentration, 2) feeding trails (*e.g.* dose-response studies) or 3) factorial modelling (Suttle, 2010; Bikker and

Jongbloed, 2014). The factorial approach estimates the total requirement on the required Zn for body functions (Bikker and Jongbloed, 2014). This approach is often used for sows, whereas feeding trials representing the empirical approach are used for growing pigs, because responses from response parameters in growing pigs becomes evident in short-term experiments. In sows, responses are less evident or lacking, as they seem to be able to use her reserves to compensate for nutrient depletion (Bikker and Jongbloed, 2014).

To determine Zn requirements, response parameters are used which level is directly related to dietary Zn intake and that reflect Zn status (Hooper *et al.,* 2009; Gibson, 2005a). These response parameters are components in tissues or body fluids (*i.e.* biomarkers) or functional indicators such as growth (de Benoist *et al.,* 2007; Gibson, 2005a; Gibson *et al.,* 2008). Many biomarkers have been used to determine Zn requirements or bioavailability or to diagnose Zn deficiency. However, Zn is required for multiple metabolic functions and metabolic or clinical symptoms to insufficient or excessive dietary Zn intake are non-specific, except growth during Zn deficiency (King, 2011). This general non-specific dysfunction makes it difficult to assess Zn status required to determine requirements.

Moreover, the presence and amount of other minerals (Ca, Fe, Cu, Cd) influence Zn absorption and affect Zn requirements consequently (Suttle, 2010). These mineral interrelationships are complex and can be antagonistic or synergistic at metabolic level and at the level of absorption (Watts, 1990). An antagonistic relationship between minerals at the absorptive level is a result of inhibited absorption (*i.e.* excessive intake of one mineral can decrease the absorption of the other mineral). Antagonistic relationships at the metabolic level are a result of the excessive intake of one mineral that interferes with the metabolic function of another mineral or contributes to the excretion of this mineral (Watts, 1990). Synergistic relationships between minerals are mostly on a metabolic level and for Zn these include K, Mg, Mn, Cr and P (Watts, 1990). For example, Zn absorption can be improved in diets with high phytate content by factors that improve P absorption (Suttle, 2010). Previous research focussed mainly on the antagonistic interactions of Zn with other minerals for Zn requirements. At excessive dietary Ca concentrations, plasma Zn concentration and Zn digestibility decreased, however, the latter is not supported in all studies in pigs (Underwood and Suttle, 1999; Suttle, 2010; Bikker and Jongbloed, 2014). Antagonistic effects between Zn and Fe are not reported in pigs (Bikker and Jongbloed, 2014). In humans, Zn stimulates Fe absorption but seems not to be essential for normal Fe levels (Scheers, 2013). Conversely, Fe supplementation with a high ratio of Fe to Zn (25:1) negatively affected Zn absorption in humans (Lönnerdal, 2000). This inhibitory effect was not present if the ratio was lower (2.5:1). Excessive dietary Cu levels decreased Zn absorption and resulted in Zn deficiency (*e.g.* parakeratosis) in both humans and pigs (Lönnerdal,

2000; Suttle, 2010; Bikker and Jongbloed, 2014). This was reversible with increased dietary Zn concentrations. Therefore, Cu does not have a negative effect on Zn absorption if dietary Zn concentration is adequate (Lönnerdal, 2000; Suttle, 2010; Bikker and Jongbloed, 2014). Contrariwise, excessive dietary Zn levels decreased Cu absorption (Watts, 1990; Suttle, 2010; Bikker and Jongbloed, 2014). Cadmium seems to accumulate in liver and kidneys of pigs at increased dietary Zn and Cu concentrations, based on their similar binding sited of metallothionein (López-Alonso, 2012a,b) and toxic levels of cadmium may inhibit Zn absorption (Lönnerdal, 2000).

Table 1.1. Recommended zinc requirements for pigs*.

Pig category	Evaluation system	Country	Recommendation
Piglets and growing- finishing pigs	NRC, 1998	USA	50-100
	NRC, 2012	USA	50-100
	INRA, 1989	France	100
	GfE, 2008	Germany	50-100
	BSAS, 2003	UK	added 60-100
Sows (gestation and lactation)	NRC, 1998	USA	50
	NRC, 2012	USA	100
	INRA, 1989	France	100
	GfE, 2008	Germany	50
	BSAS, 2003	UK	added 80
	VSP, 2015	Denmark	100
Boars	NRC, 1998	USA	50
	NRC, 2012	USA	50

* Recommendation represents total dietary Zn concentration, unless otherwise indicated.

Conclusion

Disturbances in the processes of bone remodelling, articular cartilage metabolism and horn production may lead to sow lameness. Nutrition (dietary composition, intake, and availability of the nutrients ingested) is one important factor that predisposes sow lameness in case of malnutrition. The role of proteins, lipids, and carbohydrates is not yet fully understood. Dietary mineral and vitamin deficiencies and toxicities may be detrimental to bone, articular cartilage, and horn quality.

Zinc is an essential micromineral required for multiple functions, such as optimal growth and development and reproduction. Insufficient and excessive dietary Zn intake results in clinical manifestations and dietary Zn concentrations should be adequate to minimise environmental pollution. However, Zn requirements are not completely understood and are further limited by the complexity to assess Zn status.

For this thesis, we have focused on Zn status assessment and the role of Zn in claw quality, because Zn seems to be an important factor to maintain optimal claw quality. This is based on the catalytic, structural, and regulatory Zn functions, which all may influence the processes required for horn

production. As claw lesions seem causative for lameness, improving or optimising claw quality will result in a low susceptibility for claw lesions and this may ultimately reduce the occurrence of lameness.

Supplemental information

Table 1.2. Mineral and vitamin requirements for sows and the effect of mineral and vitamin deficiencies and toxicities on lameness.

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
			Macrominerals			
Calcium (Ca)	Decreased bone mineralisation, decreased total bone mineral content, normal osteoid formation not occur or replaced by fibrous tissue, weakened and widened bone and growth plate, rickets, osteomalacia, stilted gait, lameness, posterior paralysis, enlarged and painful joints, deformation and fractures of bones, decreased Ca and P concentration in organic matrix of bone and articular cartilage (AC), decreased epidermal transglutaminase activity, production of dyskeratotic horn, bone loss	P, Mg, Fe, I, Mn and Zn deficiencies. Antagonistic to vitamin K.	8.9- 19.94 g/d	35.3-48.1 g/d	1%	Cunha, 1977; Crenshaw, 2006; McDowell, 2003; Miller *et al.*, 1962; Underwood and Suttle, 1999; Wang *et al.*, 2001
Phosphorus (P)	Decreased bone mineralisation, decreased total bone mineral content, normal osteoid formation but not mineralised, poor bone growth, bone structure abnormalities, rickets, osteomalacia, stilted gait, weakened bones	Bone loss, porous bones, calcification in soft tissues.	7.69-14.78 g/d	31.6- 40.8 g/d	1.5%	Crenshaw, 2006; Maynard *et al.*, 1979; McDowell, 2003; Underwood and Suttle, 1999; Wang *et al.*, 2001
Sodium Chloride (Na)(Cl)	Decreased protein and energy utilisation, unthriftiness	Paralysis and nervousness, muscle contraction, and transmission of nerve impulses to muscle fibres	3.15 g/d 2.52 g/d	11.93 g/d 9.55 g/d	NaCl = 8% Na = 3.14%	McDowell, 2003; NRC,1980

Table 1.2. *Continued*

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
Potassium (K)	Emaciation, inactivity, ataxia, skeletal muscle weakness, stiffness, paralysis, nervous disorders.	Muscle weakness	4.20 g/d	11.93 g/d	3%‡ Pigs tolerate up to 10x the requirements if water supply is adequate	Farries, 1958; Grim, 1980; McDowell, 2003; NRC, 1980
Magnesium (Mg)	Increased bone resorption, decreased oxidative phosphorylation, muscular twitching, muscular incoordination, reluctance to stand, stepping syndrome, weak pasterns, tetany	Only with excessive Mg supplementation levels, lethargy, locomotion disorders	1.26 g/d	3.58 g/d	0.16-0.22%	Larvor, 1983; Manicourt *et al.*, 1981; Mayo *et al.*, 1959; McDowell, 2003; Reid and New, 1997
Sulphur (S)	Deficiency is protein deficiency, affected collagen content of tendons	Reported in cows: inhibition of carbonic anhydrase, catalases, peroxidases, cytochrome C oxidase, dehydrogenases, affected oxidative metabolism and ATP production, reduced availability of other minerals, affected central nervous system	Equals the requirements for methionine		Sows provided 664 ppm S as sodium sulphate from 30d post-breeding to 28d of lactation: no reproductive problems	McDowell, 2003; Paterson *et al.*, 1979
			Microminerals			
Zinc (Zn)	Affected bone growth, affected bone size and bone strength, reduced essential fatty acid bone content, decreased bone collagen and chondroitin sulphate synthesis and turnover, reduced enzyme activity for horn production, inferior horn integrity, lameness	Interfered Cu absorption, imbalance Cu and Zn, lameness, abnormal articular cartilage (AC): thinned AC layer, patched of underlying epiphyseal bone, fractured AC surfaces, cartilage proliferation and sometimes excessive synovial fluid	210 mg/d	596.6 mg/d	1000 mg/kg DM ZnO = 2000-3000 mg/kg DM tolerated for several weeks	Hill *et al.*, 1983a; McDowell, 2003; Underwood and Sutlle, 1999; Van paemel *et al.*, 2010; van Weeren, 2006

Table 1.2. *Continued*

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
Copper (Cu)	Impaired blood vessel integrity, loss of elastic and connective tissue integrity, collagen not cross-linked and not matured, reduced leg joint rigidity, excessively flexed hocks, crooked forelegs, decreased bone deposition on calcified cartilage matrix, reduced osteoblasts activity, bone disorders (arthritis and osteoporosis), demyelination of spinal cord, reduced Cu/Zn SOD activity, nerve disorders, reduced ceruloplasmin concentration, reduced cytochrome C oxidase activity: reduced phosphorylation, decreased energy supply to keratinocytes, ataxia	Oxidative stress, convulsions, paralysis, muscular dystrophy	21 mg/d	119.32 mg/d	100 -250 mg/kg DM	Andrieu, 2008; Leeson, 2009; Linder, 1996; McDowell, 2003; O'Dell, 1984; Puls, 1984; Semevolos and Nixon, 2007; Tomlinson *et al.*, 2004; Underwood and Suttle, 1999; Van paemel *et al.*, 2010
Manganese (Mn)	Decreased GAG synthesis, decreased bone Ca conc., fat accumulation, structural changes in cell membrane, skeletal abnormalities, reduced skeletal growth, shortened & thickened bones, enlarged hock joints, muscular weakness, lameness, ataxia of newborn, impaired balance & locomotion, defects in lipid & carbohydrate metabolism	Leg stiffness, stilted gait, caused by Fe deficiency and pigs more sensitive, depressed Fe status, decreased liver Zn concentration, increased liver Cu concentration, decreased Cu absorption, neurological signs	52.49 mg/d	149.15 mg/d	1000 mg/kg DM	Grummer *et al.*, 1950; McDowell, 2003; Plumlee *et al.*, 1956; Underwood and Suttle, 1999; Van paemel *et al.*, 2010

Table 1.2. *Continued*

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
Selenium (Se)	Myodystrophy with secondary connective tissue replacement, compromised antioxidant function, difficult locomotion, reluctance to move, weakness, spraddled rear legs of newborns, unaccustomed muscle activity	Emaciation, lameness, reddened skin, hoof malformations: cracks, depressed central nervous system. In grazing livestock: neurological symptoms, titanic spasms, paralysis, abnormal posture and movement, unsteady gait, lack of thriftiness	0.31 mg/d	0.89 mg/d	4 mg/kg DM	Blaxter, 1962; EFSA, 2006; Fairweather-Tait *et al.*, 2010; Herigstad *et al.*, 1973; McDowell, 2003; Obel, 1953; Papas *et al.*, 2008; Van paemel *et al.*, 2010
Iron (Fe)	Lipid peroxidation, radial formation, Fe released in blood, reduced cellular ATP, impaired psychomotor development and cognitive performance	Peroxidative damage, especially in liver, but is dependent on vitamin E status, shivering, incoordination, titanic convulsions, posterior paralysis	168 mg/d	477.3 mg/d	3000 mg/kg DM	Andrieu, 2008; Halliwell, 1987; McDowell, 2003; Van paemel *et al.*, 2010
Chromium (Cr)	Not reported	In humans: Rhabdomyolysis	No requirements. Sows fed 0.2 ppm Cr as picolinate throughout growth and gestation had larger litter sizes and litter weights		CrO = 3000 mg/kg DM Soluble Cr^{3+} = 100 mg/kg DM‡	EFSA, 2009; Lindemann *et al.*, 1995; McDowell, 2003; Min *et al.*, 1997; Page *et al.*, 1993; Van paemel *et al.*, 2010
Molybdenum (Mo)	Not reported for sows. Mo deficiency uncomplicated by high dietary W or Cu found with diets <24ng Mo/g	In sheep: joint abnormalities, lameness, osteoporosis and spontaneous bone fractures	No requirements for sows. Rats: 0.2 ppm or goats: 0.05-0.10 ppm		150 mg/kg DM	McDowell, 2003; Van paemel *et al.*, 2010
Cobalt (Co)	Vitamin D deficiency, sensory losses. In ruminants: methylation failure, disturbed lipid metabolism	Emaciation, debility	Not required in case of adequate vitamin B_{12}		100 mg/kg DM	McDowell, 2003; Van paemel *et al.*, 2010

Table 1.2. *Continued*

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
Iodine (I)	Decreased thyroid hormone production, decreased oxidation at cellular level, muscular weakness, shortening of leg bones	In horses: hypothyroidism, weakness, lethargy, poor muscular development, osseous dysplasia of long bones, including angular deformity, tendon contraction, hyperextension, poor ossification	0.29 mg/d	0.84 mg/d	400 mg/kg DM	McDowell, 2003; Plumlee *et al.*, 1956; Van paemel *et al.*, 2010
Fluorine (F)	Possibly osteoporosis	Skeletal fluorosis, altered bone remodelling: increased bone accretion and resorption, increased total body Ca turnover, skeletal deformities: production of abnormal bone (exostosis & osteosclerosis), softening and overgrowth of bones, reduced bone collagen synthesis, delayed fracture healing, dissociation of normal osteogenesis sequence, reduced mechanical bone crystal quality, calcified ligaments, osteomalacia, joint stiffness, thickened joint, lameness, refuse to stand, moving on knees, muscle wasting, neurological symptoms, secondary and tertiary hyperparathyroidism	No requirements		150 mg/kg DM	Cerklewski, 1997; EFSA, 2004; Li, 2003; NRC, 2005; Van paemel *et al.*, 2010

Table 1.2. *Continued*

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
Aluminium (Al)		Expressed as a secondary P deficiency, reduced F utilization, nervous system most sensitive, oxidative stress, incoordination of hind legs, inhibit formation of bone phosphates, decreases antioxidant enzymes (*e.g.* Mn-SOD), thereby altering the ability for oxidative damage protection. In humans: osteomalacia and aplastic bone disease	Goats and other animals: < 10 mg Al/kg DM		1000 mg/kg DM‡	Foy and Brown, 1964; Kawahara *et al.*, 2007; Lieberherr *et al.*, 1982; McDowell, 2003; Struys-Ponsar *et al.*, 2000; Van paemel *et al.*, 2010; Verstraten *et al.*, 2008
Arsenic (As)	Decreased S-adenosylmethionine (SAMe)	Uncoordinated and staggered gait, swaying, microcirculation abnormality	No requirements. In diets 0.85 mg/kg DM		30 mg/kg DM‡	Fowler *et al.*, 2007; McDowell, 2003; Nielsen, 1996; Uthus, 2003; Van paemel *et al.*, 2010;
Boron (B)	Impaired cellular Ca transport	Inflammation and edema in leg and dewclaw, decreased inflammatory response and thyroid hormone production, lethargy, In cows: decreased plasma P concentration. In poultry: curled toe paralysis	No requirements. In humans: 1-3 mg/d is beneficial for bone and brain health		150 mg/kg DM‡	Armstrong *et al.*, 2001; Armstrong *et al.*, 2002; Devirian and Volpe, 2003; McDowell, 2003; Nielsen, 1997; Nielsen, 2008; Van paemel *et al.*, 2010
Bromine (Br)	Not reported	Disturbed thyroid function. In humans and rats: altered central nervous system function, influenced I metabolism, disturbed coordination, tremor, depressed tendon reflexes.	No requirements. In humans 0.4 mg/kg BW is acceptable		200 mg/kg DM	McDowell, 2003; Nielsen, 1996; Van Leeuwen *et al.*, 1987; Van paemel *et al.*, 2010

Table 1.2. *Continued*

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
Cadmium (Cd)	Not reported	Enlarged joints, decreased Ca absorption, decreased bone mineralisation, osteomalacia, osteoporosis	No requirements. In humans: provisional weekly intake 0.5-7 µg/kg BW		10 mg/kg DM	EFSA, 2009; McDowell, 2003; Nordberg *et al.*, 2007; Powell *et al.*, 1964; Rimbach *et al.*, 1996; Van paemel *et al.*, 2010
Cerium (Ce)	Not reported	Not reported	No requirements. Daily intake between 12-120 mg/d		100 mg/kg DM	NRC, 2005, Redling, 2006; Van paemel *et al.*, 2010
Germanium (Ge)	Altered bone and liver mineral composition	Not reported	No requirements		No MTL	McDowell, 2003; Seaborn & Nielsen, 1994
Lanthanum (Lt)	Not reported	Uncommon	No requirements		No MTL, 100 mg/kg DM should be safe	Redling, 2006; Van paemel *et al.*, 2010
Lead (Pb)	Not reported	Muscle twitching and weakness, ataxia, joint stiffness, paralysis, demylination, slow conductance velocity, lethargy, neurological impact	No requirements		10 mg/kg DM	EFSA, 2004; McDowell, 2003; Van paemel *et al.*, 2010
Lithium (Li)	Not reported for sows	Neuromuscular symptoms, depressed thyroid function. In cattle: ataxia	No requirements		25 mg/kg DM	Aral and Vecchio-Sadusm 2008; Gitlin, 1999; Nielsen, 1996; Schrauzer, 2002; Van paemel *et al.*, 2010

Table 1.2. *Continued*

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
Mercury (Hg)	Not reported	Abnormal gait & inactivity with inorganic Hg, impaired brain and nervous system functioning with organic Hg, ataxia, muscle spasms, paralysis, hind limb crossing with methyl Hg. Promote lipid peroxidation, complex Se and inhibit glutathione peroxidase formation. Liver failure is the endpoint for pigs	No requirements		Inorganic Hg =0.2 mg/kg DM Organic Hg = 2 mg/kg DM‡	Berlin *et al.*, 2007; Chang *et al.*, 1977; Gonzalvo *et al.*, 1997; McDowell, 2003; Van paemel *et al.*, 2010
Nickel (Ni)	Altered distribution and proper functioning of other nutrients, including Zn, Fe and Ca incorporation in the skeleton, skeletal lesions	Lethargy. In rats: ataxia	50-200 ppb		250 mg/kg DM	McDowell, 2003; Nielsen and Shuler, 1979; Nielsen, 1996, Nielsen, 1997; Van paemel *et al.*, 2010
Rubidium (Rb)	Not reported for sows	In rats: extreme nervousness and convulsions	No requirements		No MTL	Van paemel *et al.*, 2010
Silicon (Si)	Abnormal shaped bones and cartilaginous tissue in chicks, rats and calves, decreased GAG and collagen concentration in cartilage, altered chemical bone composition, smaller long bone joint and reduced strength, decreased bone hydroxyproline and ALP activity, abnormal collagen formation	In cattle: kidney problems	No requirements		No MTL	Frantz, 2006; McDowell, 2003; Uthus and Seaborn, 1996; Van paemel *et al.*, 2010
Silver (Ag)	Not reported	Necrosis of bone marrow	No requirements		100 mg/kg DM‡	Van paemel *et al.*, 2010

Table 1.2. *Continued*

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
Strontium (Sr)	Not reported	Disturbed Ca metabolism, rickets if dietary Ca is inadequate. In rats: increased ALP activity. In poultry: decrease bone organisation and mineralisation	No requirements		2000 mg/kg DM	Van paemel *et al.*, 2010
Tin (Sn)	Not reported	Might affect Cu, Zn and Fe metabolism and alters Ca metabolism with inorganic Sn, edema of central nervous system with organic alkyl Sn	No requirements		100 mg/kg DM‡	McDowell, 2003; Schwarz, 1974; Van paemel *et al.*, 2010
Vanadium (V)	In goats: skeletal deformations in front legs, thickened front leg joints in goats, impaired bone tissue metabolism and Fe metabolism	In ruminants: nervous disturbance, emaciation, paralysis of hind legs, ataxia	No requirements		10 mg/kg DM‡	Anke *et al.*, 1989; Hopkins and Mohr, 1974; McDowell, 2003; Nielsen, 1997; Van paemel *et al.*, 2010
		Vitamins				
Vitamin A	Poor bone development, poor horn growth, poor horn calcification	Increased bone resorption and/or bone formation, bone loss, spontaneous bone fractures	8398 IU§	11932 IU§		Binkley and Krueger, 2000; Coates *et al.*, 1998; Kellon, 2008; Thompson *et al.*, 1967; Waldenstedt, 2006
Vitamin B	Impaired bone metabolism, bone abnormalities, weak, brittle and necrotic claws, heel erosion and cracks, neurological lesions		Biotin: 0.42 mg/d Niacin (available): 21 mg/d	Biotin: 1.19 mg/d Niacin (available): 59.66 mg/d		Bryant *et al.*, 1985; Cagnacci *et al.*, 2003; Waldenstedt, 2006

Table 1.2. *Continued*

Nutrient element	Deficiency	Toxicity	Requirements*		Maximum tolerance levels (MTL)†	References
			Gestation	Lactation		
Vitamin C	Poor collagen synthesis					Foulds, 1993; Mahan and Arlin, 1992; Percival, 1997; Rennie and Whitehead, 1996; Waldenstedt, 2006; Weiser *et al.*, 1988; Whitehead *et al.*, 1994
Vitamin D	Decreased bone mineralisation, bone structure abnormalities, sensory losses, decreased Ca and P availabilities	Calcification of soft tissue, affected joints, disturbed AC growth, Ca deposition, cellular degeneration, bone demineralisation, thinning of bones	1680 IU§	4773 IU§		Clarke, 2008; Goff, 2010; McDowell, 2003; Tomlinson *et al.*, 2004; Underwood and Suttle, 1999
Vitamin E	Increased lipid peroxidation, decreased energy production required for keratinisation process, increase occurrence of lesions, equals selenium deficiency symptoms, neuropathy	Impaired vitamin D utilization	92.4 IU§	262.5 IU§		McDowell, 2003; Percival, 1997; Sokol, 1996; Tomlinson *et al.*, 2004; Waldenstedt, 2006

* The mineral and vitamin requirements for sows are based on a daily feed intake + wastage (estimated wastage is 5%) of 2210 g/d during gestation and 6280 g/d during lactation (NRC, 2012)

† The maximum tolerance levels are based on NRC, 2005.

‡ The maximum tolerance levels for pigs (including sows) are derived from interspecies extrapolation (NRC, 2005; Van paemel *et al.*, 2010).

§ 1 IU vitamin A= 0.30 µg retinol or 0.344µg retinyl acetate. 1 IU vitamin D= 0.025 µg and 1IU vitamin E= 0.67 mg of D-α-tocopherol or 1 mg of DL-α-tocopheryl acetate.

Chapter 2

Research objectives

Research objectives

Claw lesions are a common multifactorial disorder in sows that cause animal discomfort and economic losses (Heinonen *et al.*, 2013; Chapter 1). Malnutrition is an important predisposing factor related to claw lesions: an interrupted nutrient supply to the claw causes a deteriorating claw quality, in which the claw becomes more susceptible to claw lesion development. Besides the importance of biotin and sulphur-containing amino acids to maintain claw quality, the role of Zn seems important, because of the catalytic, structural, and regulatory functions of Zn in horn production; however, its role in claw health is inconclusive. Zinc status is difficult to assess and it is not clear which biomarker is most suited throughout the reproductive cycle. Biomarkers for Zn status might show decreases at particular phases of reproduction, but it is yet unknown whether promoting Zn absorption through higher dietary Zn concentration or other means will lead to improved Zn status at those phases, and if they will serve to maintain claw quality. The overall aim of this thesis was therefore to evaluate whether dietary strategies can improve Zn status in sows and if this exerts changes in claw quality.

The following objectives were defined to:

- evaluate the suitability of the most widely used biomarkers for Zn status assessment in production animals (Chapter 3)
- determine possible fluctuations of Zn status biomarkers throughout a reproductive cycle in sows (Chapter 4)
- determine the effect of protein and Zn source on Zn status and Zn bioavailability in sows (Chapter 5)
- determine the effect of dietary Zn concentration on Zn status biomarkers and claw quality in weaned piglets and in sows (Chapter 6)

Chapter 3

Zinc status assessment

Review: Assessing zinc status in production animals: choosing the appropriate biomarkers.

M.M.J. van Riet, G.P.J. Janssens, P. Bikker, M. Aluwé, E. Nalon, F.A.M. Tuyttens, D. Maes, and S. Millet.

Submitted.

Abstract

Zinc is an essential micromineral for humans and for production animals alike. Zinc is involved in many processes and is homeostatically tightly regulated. During insufficient or excessive dietary Zn intakes relative to Zn requirements, complex physiological adjustments occur in the absorption and excretion of Zn. This requires an adequate assessment of dietary Zn intake and the different Zn concentration levels in the body to determine Zn status. Such assessment is difficult, however, and probably differs between humans and production animals. Important biomarkers have been described in human studies; here, we review the same biomarkers for their suitability to assess Zn status in production animals during growth and reproduction. This can support the selection of appropriate biomarkers for Zn status assessment to diagnose Zn deficiency or to determine Zn requirements and Zn bioavailability as common study objectives. This review shows that dietary Zn intake, as exposure biomarker, in most production animals are adequate, but the possibility to reduce Zn levels in feed should be studied, preferably in combination with other biomarkers to assess fluctuations in Zn status. Different responses of the biomarkers to dietary Zn intake, especially in body tissues, were found between humans and production animals, and also among animal species. In general, plasma Zn concentration seems to be an appropriate marker for assessment of Zn requirements in most cases. Zinc concentration in body tissue is less suitable, except for bone Zn concentration in poultry. Plasma alkaline phosphatase (ALP) does not appear to be sensitive to dietary Zn intake and the use of metallothionein (MT) requires further research. As a functional indicator, the daily growth of production animals can be used as a response criterion for Zn status assessment. Therefore, Zn status assessment in production animals should be based on a well-considered choice of the most appropriate biomarker depending on species and study objective.

Introduction

Zinc, an essential micromineral in humans and animals, has important structural, regulatory and catalytic functions. It is involved in many enzymatic processes, growth and development, wound healing, skin, hair and nail/claw quality, bone metabolism, immune function, gene expression, hormone regulation and lipid metabolism (McDowell, 2003; Peters, 2006; King, 2011; Naithani *et al.,* 2014). Unlike other microminerals, the availability of a functional reserve or Zn store in the body from which Zn can be readily utilised is limited. Insufficient or excessive dietary Zn intake relative to Zn requirements results in complex physiological adjustments to maintain homeostasis (King, 1990 and 2011; Lowe *et al.,* 2009; Naithani *et al.,* 2014). Zinc homeostasis is strictly regulated through absorption and (endogenous) excretion in the small intestine and can respond to alterations in dietary Zn intake until its homeostatic capacity is exceeded (King *et al.,* 2000; Lowe *et al.,* 2009; Naithani *et al.,* 2014). Depending on the level of insufficient or excessive dietary Zn intake, adjustments in Zn concentration in body tissues and fluids occur rapidly, but also functional losses (depressed growth or reproductive failure) and clinical signs (loss of appetite, anorexia and parakeratosis) become visible over time, even leading to death in severe cases (Suttle, 2010). This emphasises the need to understand the Zn requirements for optimal health and the mechanisms involved during insufficient or excessive dietary Zn intake (Lowe *et al.,* 2009).

Assessing Zn status is difficult, however, because of the lack of consensus of the most appropriate biomarker (no golden standard) that reflects Zn status optimally in different experimental settings (Kincaid, 1999; Wood, 2000; Hambidge, 2003). Biomarkers are components in tissues or body fluids whose level is directly related to dietary Zn intake over a short, medium, or long-term period; they reflect changes in Zn homeostasis and thus Zn status (Hooper *et al.,* 2009; Gibson, 2005a).

In human studies, many biomarkers have been used. Those physiological processes may differ from growing or breeding production animals, however, the applicability and implications of using human Zn status biomarkers in production animals are not well understood.

Severe Zn deficiency is seldom seen in production animals (Wood, 2000; McDowell, 2003; Peters, 2006), because Zn is generally supplemented to the diet above the species-specific Zn requirements to prevent production losses (*e.g.* depressed growth or reproductive failure) (Creech *et al.,* 2004; Blanco-Penedo *et al.,* 2009). However, even a marginal Zn deficiency may affect growth and reproduction in the absence of clinical signs, highlighting the need for specific biomarkers (Peters, 2006; Gibson *et al.,* 2008). Conversely, excessive supplementation of Zn above the species-specific requirements can become toxic for animals and contributes to the excretion of excessive Zn into the environment, thereby negatively affecting soil and water conditions (Creech *et al.,* 2004; Jongbloed *et al.,* 2004; Spears and Hansen, 2008). Determining optimal Zn requirements and using highly

available Zn sources may reduce Zn excretion. Detection of marginal Zn deficiency (specific biomarkers), determination of Zn requirement (sensitive biomarkers) and the use of more available Zn sources are common objectives in studies on production animals, where growth, reproduction and ecological sustainability of the production process are all important to the production process (Kincaid, 1999; Spears and Hansen, 2008).

In this review, we evaluate several biomarkers described in studies that address human responses to different levels of dietary Zn intake. Can these biomarkers be used to 1) detect Zn deficiency and 2) determine Zn requirements and availability in production animals during growth and reproduction? The included studies report on responses to insufficient dietary Zn concentrations (*i.e.* below recommended requirements) as well as adequate and excessive Zn concentrations, where Zn is supplemented at or above physiological concentrations up to 200 mg added Zn/kg diet. Biomarkers responding to dietary Zn intake are suitable for Zn status assessment, but are only suitable to determine Zn requirements if a plateau is formed at a specific dietary Zn concentration (*e.g.* no continuing accumulation of surplus Zn).

The (potential) biomarkers are divided into three category's:

1) exposure: dietary Zn intake;
2) status: body tissues, plasma Zn, enzymes (alkaline phosphatase (ALP)), proteins (metallothionein (MT)), excretion; and
3) functional indicators which are not components in tissues or body fluids but do reflect the functional consequences of Zn deficiency: prevalence of diarrhoea, growth performance, milk/egg production and reproduction.

Per (potential) biomarker, the responses in growing production animals are described first, followed by the same responses in breeding production animals.

Exposure biomarkers

Dietary Zn intake is the most commonly used biomarker within this group. Dietary concentrations may provide information regarding the adequacy of dietary Zn intake (de Benoist *et al.,* 2007), resulting in the proportion or probability of a population with dietary Zn intake below the Zn requirements (de Benoist *et al.,* 2007). This estimation of the risk of dietary Zn deficiency in a human population is a recommended biomarker by WHO/UNICEF/IAEA/IZINCG and validated by IZINCG for adults and children (de Benoist *et al.,* 2007; Gibson *et al.*, 2008). It is used to assess the impact of intervention studies (de Benoist *et al.,* 2007).

Humans and laboratory animals

To our knowledge, dietary Zn intake is the main criterion used to detect Zn deficiency in humans, because insufficient dietary Zn intake is a major factor associated with Zn deficiency (Gibson *et al.*, 2008; Roohani *et al.*, 2013). Exacerbating factors are high physiological requirements, presence of disease, excessive Zn losses and use of certain therapeutic drugs (Gibson *et al.*, 2008). Consequently, growth rate in children and Zn excretion in adults may be adjusted to maintain Zn homeostasis (Gibson *et al.*, 2008).

Production animals

The use of dietary Zn intake as single biomarker for Zn status assessment in production animals is not common. Although species-specific Zn requirements are widely documented, their recommendations are based on a limited and dated number of studies, especially for cattle and sows. Zinc inclusion in the diet generally exceeds the nutrient requirements and Zn deficiency is less likely to occur in production animals (Creech *et al.*, 2004; Peters, 2006; Blanco-Penedo *et al.*, 2009). Marginal Zn deficiency could conceivably occur under certain conditions such as low feed intake, low addition of minerals in premix, low availability of minerals, animals kept on pasture, extensive or organic management systems and poor feed formulation (Peters, 2006; Humann-Ziehank *et al.*, 2008; Blanco-Penedo *et al.*, 2009). Especially for cattle, diets may not always contain sufficient microminerals to meet their demands (Pogge *et al.*, 2012).

Suitability of dietary zinc concentrations for zinc status assessment

Relying only on dietary Zn intake to diagnose Zn deficiency is difficult, because knowledge of Zn requirements in production animals is inadequate (Mills *et al.*, 1967; Jongbloed *et al.*, 2010; Bikker and Jongbloed, 2014) and Zn requirements are difficult to determine (Jongbloed *et al.*, 2004). Furthermore, the available dietary Zn concentration is influenced by several factors, such as dietary composition (protein source) and availability (high levels of phytate). For production animals, growth performance is more frequently described as indicator (see section "functional indicators" below). Therefore, assessment of dietary Zn intake should preferably be used in combination with performance characteristics or other Zn status biomarkers to ratify the observed Zn status and to estimate the animal's Zn requirements (Kincaid, 1999; Jongbloed *et al.*, 2004).

Status biomarkers

Status biomarkers represent objective and quantitative response criteria to assess Zn status of an individual or population. The aim is either to indicate the presence and severity of Zn deficiency (de

Benoist *et al.,* 2007) or to support estimations of Zn requirement and Zn bioavailability (Jongbloed *et al.,* 2004). Within this group of biomarkers, concentrations of Zn in body tissues and plasma, plasma ALP concentration, MT concentration and faecal and urinary Zn excretion are evaluated.

Zinc concentration in body tissues

Body Zn content appears to be under close homeostatic control by regulation of Zn absorption and excretion (Jackson and Lowe, 1992). Body tissues respond to changes in dietary intake by either increasing or decreasing their Zn concentration, but not all tissues may respond in the same way to changes in Zn concentration (see below).

Humans and laboratory animals

Compensation for a low dietary Zn intake occurs by mobilising and releasing Zn from bone and liver tissue to support and maintain Zn concentration in other tissues (Jackson *et al.,* 1982; King, 1990; King *et al.,* 2000; Hess *et al.,* 2007). Bone and liver Zn concentration decrease during a period of low dietary Zn intake, whereas muscle Zn concentration seems to increase in these periods (Jackson *et al.,* 1982; King, 1990; Hess *et al.,* 2007).

When dietary Zn supply exceeds the dietary requirement, the intestinal uptake can be decreased to reduce the translocation of Zn from the intestinal mucosa into the body. In rats fed excess Zn, the total body Zn content remained constant, whereas faecal Zn excretion increased (Buckley and D'Mello, 2000). Although total body Zn did not seem to increase, the Zn content of specific tissues such as bones, muscles, skin and hair increased due to the deposition of Zn in those relatively slow-responding exchangeable Zn pools (King, 1990; Hess *et al.,* 2007). The highest accumulation was found in the pancreas, liver and kidneys (McDowell, 2003).

Production animals

The Zn content of certain body tissues is also adapted in production animals to compensate for insufficient or excessive dietary Zn intake. The responses of liver, kidney, pancreas and bone tissue are described. These tissues are evaluated instead of other tissues such as skin, muscle and hair, because these organs and bone tissue are more closely related to Zn metabolism.

Liver. In calves, liver Zn concentration only increased when pharmacological dietary Zn concentrations (≥200 mg added Zn/kg DM) were supplied (Table 3.1) (Miller, 1970 and 1979; Kincaid, 1999; Hosnedlova *et al.,* 2007). Based on one study in lambs, liver Zn concentration in this species increased with incremental dietary Zn concentrations above 20 mg added Zn/kg (Table 3.2).

Similarly, liver Zn concentration increased in piglets with increasing dietary Zn concentrations. However, while some studies in piglets found a plateau for liver Zn concentrations, other studies found a further increase in concentration (Table 3.3; Bikker and Jongbloed, 2014). In broilers, results are contradictory (Table 3.4). Liver Zn concentration increased in two studies (Bartlett and Smith, 2003; Sunder *et al.,* 2008), while it decreased in another study where Zn was supplemented in combination with other microminerals (Bao *et al.,* 2007), or a response was found depending on phytase level (Zaghari *et al.,* 2015). Miller (1979) and Spears and Hansen (2008) also indicated that liver Zn concentration did not respond linearly to increased dietary Zn concentrations in poultry as it did in piglets.

In breeding production animals such as mature cattle, liver Zn concentration marginally decreased during low dietary Zn intake (Miller, 1979). Zinc concentration in liver and other body tissues (except rib cartilage) did not respond to increased dietary Zn intake as shown in a study using lactating dairy cattle (Neathery *et al.,* 1973) (Table 3.1). However, in ewes, sows and in laying hens, liver Zn concentrations were higher in supplemented compared to non-supplemented control animals (Table 3.2-3.4, sows: Jongbloed *et al.,* 2010), indicating a species-specific response as the dietary Zn concentrations were in a similar range.

Kidney and pancreas. In calves, Zn concentration in kidney and pancreas only increased at pharmacological dietary Zn concentrations (≥200 mg added Zn/kg DM) (Table 3.1) (Miller, 1970 and 1979; Kincaid, 1999; Hosnedlova *et al.,* 2007). In lambs, kidney Zn concentration did not respond to dietary Zn intake (Table 3.2). In broilers, kidney and pancreas Zn concentration increased with increasing dietary Zn concentrations. However, one study on broilers found a plateau for pancreas Zn concentration up to 40 mg added Zn/kg diet (Table 3.4).

In kidney and pancreas of supplemented ewes, Zn concentrations increased, but not in cattle (Table 3.1 and 3.2). Similarly, kidney and pancreas Zn concentrations rose in supplemented laying hens but not in sows (Table 3.3 and 3.4).

Bone. In piglets, fattening pigs and broilers, Zn concentration in bone increased with increasing dietary Zn concentrations (Table 3.3 and 3.4). No plateau was reported in piglets, but a plateau was observed in fattening pigs (plateau at 20 mg added Zn/kg diet) and in most studies on broilers (plateau between 40 and 60/80 mg added Zn/kg diet) (Table 3.4). Possibly the difference in age between piglets and fattening pigs and the Zn supply in relation to the Zn requirement explains the plateau found in fattening pigs but not in piglets.

In breeding production animals, Zn concentration in bone of bulls and gravid ewes showed no difference between the non-supplemented control and Zn supplemented group (Table 3.1 and 3.2) (Illek, 1990; Hosnedlova *et al.*, 2007). However, in two sow studies, bone Zn concentration did increase after Zn supplementation compared to non-supplemented control animals (Table 3.3) (Jongbloed *et al.*, 2010).

Suitability of body tissue zinc concentrations for zinc status assessment

In body tissues, the response of Zn content to dietary Zn intake in humans differs from that of production animals. Among production animals responses differed depending on animal species as well as age.

In both growing and breeding cattle, body tissues did not respond in a dose dependent manner to dietary Zn intake and thus seem to be less suitable to determine Zn requirements. One study in ewes and one study in lambs report, however, that liver and pancreas Zn concentrations may be more sensitive biomarkers to determine Zn requirements than bone and kidney, because liver and pancreas seem to respond better to dietary Zn intake. For diagnosing Zn deficiency in lambs, liver seems to be insufficiently strong as a biomarker, because it responded only above 20 mg added Zn/kg DM in one study (Pal *et al.*, 2014). However, more research is required to confirm this postulation in sheep.

Compared to ruminants, Zn concentration of body tissues in pigs and poultry responded more sensitively to dietary Zn intake, making these body tissues suitable for Zn status assessment. Suitability differs according to the study objective: Zn deficiency, requirements or bioavailability. The liver seems sensitive to dietary Zn intake in both pigs and poultry. Despite comparable dietary Zn concentrations in piglet studies, the results were contradictory between studies and a plateau was not consistently observed. These contradictory observations warrant caution when using liver Zn to determine Zn requirements or deficiency diagnosis. In pigs, bone seems to accumulate Zn independent of increased dietary Zn intake, but this is not the case in poultry. Therefore, bone Zn concentration can be suitable to determine Zn requirements in poultry. Studies, which did not report a plateau, used older broilers or used only 1 supplementation level compared to the non-supplemented controls. The use of body tissue as biomarker for Zn status assessment in sows and laying hens should be investigated further, including several dietary Zn inclusion rates.

Spears and Hansen (2008) and Jongbloed (2010) have described the use of body tissues as biomarker for Zn bioavailability studies in production animals, but the impact seems species- and tissue-specific as well as dependent upon dietary Zn concentration. In cattle, Zn body tissue concentrations seem to be unsuitable as a biomarker. In pigs and poultry, Zn concentrations in

organs seem less suitable compared to the concentration in bone. The ability of bone to accumulate Zn at periods of increased dietary Zn intake makes it ideal to determine Zn bioavailability.

Table 3.1. Response of zinc status biomarkers in cattle fed varying dietary zinc concentrations[*].

Category	Basal diet (mg Zn/kg DM)	Intervention (added Zn)	Responses to Zn addition[†]											References
			Body tissues						Blood			Excretion		
			Liver	Pancreas	Kidney	Intestine	Muscle	Bone	Zn	ALP	MT	Faeces	Urine	
Calves	20	0, 50, 500	+ > 500		+ > 500		=	=	+ > 500					Kincaid and Cronrath, 1979
Calves	60	0, 150, 300	+ at 300						+ at 300					Kincaid *et al.*, 1997
Calves	33 mg/kg	0, 200, 600	+ > 200	+> 600	+> 600	+ > 200 & > 600	=	=	+ > 600			+ > 200 & 600 (wk 2 & 3)	=	Miller *et al.*, 1970
Calves	1.2	0, 2, 8, 25							+ < 25					Mills *et al.*, 1967
Calves	20	0, 200	=	=	=		=	=	=					Rojas *et al.*, 1996
Calves	28	0, 20, 500	+ at 500		+ at 500	+ at 500		+ at 500 bone shaft	+ at 500	=	+ at 500 liver MT			Wright and Spears, 2004
Heifers	23.8	0, 25							Tended to + at d112	+ d42, 112				Spears, 1989
Steers	60.1	0, 1 mL/45 kg BW (60 mg Zn/mL)	+						+					Pogge *et al.*, 2012

Table 3.1. *Continued*

Category	Basal diet (mg Zn/kg DM)	Intervention (added Zn)	Responses to Zn addition[†]											References
			Body tissues						Blood			Excretion		
			Liver	Pancreas	Kidney	Intestine	Muscle	Bone	Zn	ALP	MT	Faeces	Urine	
Bulls	35	0, 10	=				=	=	=	=	= in tissues			Kessler *et al.*, 2003
Cows	154.8 mg Zn/cow	0, 910.2 mg/cow							+					Anton *et al.*, 2013
Cows	31	0, 6.1, 18.3							=					Lethbridge, 2009
Cows	16.6	0, 22.9	=	=	=	= except some parts of intestine (+)	=	= except rib cartilage (+)				+		Neathery *et al.*, 1973
Cows	37.6	0, 40							=	=				Wang *et al.*, 2013
Cows	36	0, 60, 300							=					Cope *et al.*, 2009

Zn, plasma Zn; ALP, plasma Alkaline phosphatase; MT, Metallothionein; DM, dry matter basis.

[*] Responses to Zn addition in the value of the observed biomarker are indicated as no effect (=), a decrease (-), or an increase (+), until (<) or beyond (>) a specific Zn addition.

[†] Responses are represented related to the added Zn concentration to the diet and not total dietary Zn concentration.

Plasma zinc concentration

In blood, total Zn concentration can be determined in whole blood, plasma or serum, erythrocytes, platelets and leukocytes. Plasma or serum Zn concentration is the most widely used biomarker to assess Zn status in human populations and is currently the only recommended biomarker for this purpose (de Benoist *et al.,* 2007; Hess *et al.,* 2007; Gibson *et al.,* 2008).

Zinc in plasma is predominantly transported while loosely bound to albumin (about two-thirds) and to a lesser extent to α2-macroglobulin and high molecular weight binding ligands (McDowell, 2003; Gibson *et al.,* 2008). All absorbed Zn passes through the plasma before it reaches the tissues. Plasma Zn is homeostatically regulated within a particular range (in humans in fasting state: 78 to 100 μg/dL) by the Zrt- and Irt-like proteins (ZIP) and Zn transporter (ZnT) protein families, and therefore subject to adaptive responses at different levels of dietary Zn intake, even below or above the Zn requirements (Hess *et al.,* 2007; Gibson *et al.,* 2008; Naithani *et al.,* 2014). The influx as well as the efflux occur rapidly to maintain a constant concentration of plasma Zn (King *et al.,* 2000), thereby providing a circulatory route for Zn and other minerals to various body tissues (Lowe *et al.,* 2009).

Generally, Zn concentration in plasma and serum (plasma without fibrinogen or other clotting factors) respond similarly to varying dietary Zn concentrations (Jongbloed *et al.,* 2004; Hess *et al.,* 2007), indicating that these fibrinogen and clotting factors have no effect on variation in total plasma Zn concentrations. Therefore, plasma and serum concentrations are considered as the same biomarker.

Humans and laboratory animals

In depletion/repletion studies in humans, plasma Zn concentration decreased considerably (between 25 and 88% within 2 to 9 weeks) with severe restriction of dietary Zn intake (Hess *et al.,* 2007; Gibson *et al.,* 2008; King, 2011). This resulted in an increased fractional turnover rate, a decreased quantity of Zn distributed into various tissues associated with alteration in total body Zn content and tissue dysfunction (King *et al.,* 2000; Lowe *et al.,* 2004; Gibson *et al.,* 2008). After receiving the Zn repletion diet, the plasma Zn concentration returned rapidly to baseline levels (Hess *et al.,* 2007; Gibson *et al.,* 2008).

Plasma Zn responses in individuals are inconsistent during moderate dietary Zn restrictions, due to the effective homeostatic mechanism to maintain plasma Zn concentrations (Hess *et al.,* 2007; Gibson *et al.,* 2008; Naithani *et al.,* 2014). Plasma Zn concentrations decrease only slightly after a

prolonged period of Zn restriction, in some cases with no change at all (Hess *et al.,* 2007; Gibson *et al.,* 2008).

A significant and positive plasma Zn response was found during long-term Zn supplementation in children and during short-term Zn supplementation in well-nourished healthy adults. Supplementation of Zn also alleviated the usual decline in plasma Zn concentration in pregnant women (Hess *et al.,* 2007; Brown *et al.,* 2002; Gibson *et al.,* 2008). A plateau was reported at a dietary Zn intake of approximately 20-30 mg/d, reflecting the fractional Zn absorption during Zn supplementation (Gibson *et al.,* 2008; King, 2011).

Production animals

Plasma Zn concentration in growing production animals (calves, lambs, doelings and piglets) were reduced during a period of low dietary Zn intake (Table 3.1-3.3) (Swinkels *et al.,* 1996; Spears and Hansen, 2008). Calves and lambs responded to low dietary Zn intake within one week. Generally, increased dietary Zn concentration results in an increased plasma Zn concentration (Bikker and Jongbloed, 2014), although some studies in lambs, calves, or broilers found no effect on plasma Zn concentration or only at pharmacological dietary Zn concentrations (300 mg added Zn/kg DM) (Table 3.1-3.4). Furthermore, the plasma Zn response to Zn supplementation is different between studies and between species: some studies in 1-year-old goats and lambs only found an increased plasma Zn concentration above 30-45 mg added Zn/kg DM or 20 mg added Zn/kg DM, respectively. In piglets and fattening pigs, a plateau was found between 20 and 60 mg added Zn/kg, whereas others did not find a plateau, or only found an increased plasma Zn concentration if phytase was not added to the diet.

In breeding production animals (cows, ewes and sows), plasma Zn concentration was lower during low dietary Zn intake during gestation and lactation (Table 3.1-3.3) (Miller, 1979; Kincaid, 1999; Pond *et al.,* 1995). However, one study in sows showed only a marginal decrease in plasma Zn concentration during gestation and a significant decrease during lactation (Table 3.3). In boars, plasma Zn concentration decreased when they were fed low dietary Zn concentrations as soon as 36 hours from onset of a severe dietary Zn restriction (Liao *et al.,* 1985). Increasing dietary Zn concentration resulted in increased plasma Zn concentration in sheep (Kincaid, 1999), sows and laying hens, but plasma Zn seems to be less responsive in cattle, albeit based on a limited number of studies (Table 3.1-3.4).

Suitability of plasma zinc concentrations for zinc status assessment

Plasma Zn responses to dietary Zn intake are similar between humans and some production animals, although differences among species exist.

In cattle, plasma Zn concentration seems to have a low responsiveness; furthermore, the results are contradictory which hampers the evaluation of suitability of plasma Zn as biomarker for Zn status assessment in cattle. However, Spears and Hansen (2008) suggested that plasma Zn may be a good biomarker to determine Zn bioavailability. Indeed, some studies do report that Zn source influenced plasma Zn concentrations in cattle (Sobhanirad and Naserian, 2012; Wang *et al.*, 2013).

In sheep and goats, plasma Zn concentration seems less suitable to determine Zn requirements and diagnose Zn deficiency, as no plateau was reported or responses were only found above a certain dietary Zn concentration.

Different responses in pigs and poultry suggest that plasma Zn concentration seems sensitive enough to determine Zn requirements and to evaluate bioavailability below Zn requirements in piglets, but not in poultry. The dietary Zn concentration was similar between poultry studies, but study duration and addition of other microminerals to the diet differed and may have interfered with the plasma Zn responses found.

Plasma Zn concentration as a diagnostic biomarker for Zn deficiency in production animals seems to be inappropriate, because confounding factors such as (heat) stress, age, reproduction, fasting state and presence of infection or disease are reported to interfere (*i.e.* increase or decrease) with plasma Zn concentrations (King, 2011), affecting the specificity of plasma Zn. Its use may be more valuable when low plasma Zn concentrations are repeatedly observed over time as suggested by Mills *et al.* (1967). However, more research is required for all species.

Table 3.2. Response of zinc status biomarkers in goat and sheep fed varying dietary zinc concentrations[*].

Species	Basal diet (mg Zn/kg DM)	Intervention (added Zn)	Responses to Zn addition†										References
			Body tissues						Blood			Excretion		
			Liver	Pancreas	Kidney	Intestine	Muscle	Bone	Zn	ALP	MT	Faeces	Urine	
Goats														
Kids	15	0, 65							+					Chhabra and Arora, 1985
1-y-old	22.3	0, 15, 30, 45							+ > 30, 45					Jia *et al.*, 2008
1-y-old	22	0, 40, 120, 200							+					Puchala *et al.*, 1999
Dairy	78	0, 1 g/d										+		Salama *et al.*, 2003
Sheep														
Lambs	1.2	0, 2, 7.1, 24							- < 20					Mills *et al.*, 1967
Lambs	35 mg/kg	0, 20, 40	+ > 20		=		+ > 40		+ up to 90 d	+ > 40 to 90 d				Pal *et al.*, 2014
Lambs	2.8	0, 5 (d0-42), 15 (d42-56)								+	+ after 28 d			Spears, 1989
Lambs	4	0, 10, 17, 29							+ > 23 mg					White *et al.*, 1994
Ewes[‡]	<1 mg/kg + 16-20 mg/kg	0, 20	+	+	+		+	=	+	+				Apgar and Fitzgerald, 1987
Ewes[‡]	<1 mg/kg + 15-20 μg/g DM	0, 100							+					Apgar and Travis, 1979

Zn, plasma Zn; ALP, plasma alkaline phosphatase; MT, metallothionein; DM, dry matter basis.

[*] Responses to Zn addition in the value of the observed biomarker are indicated as no effect (=), a decrease (-), or an increase (+), until (<) or beyond (>) a specific Zn addition.

[†] Responses are represented related to the added Zn concentration to the diet and not total dietary Zn concentration.

[‡] Studies provided a semipurified diet with <1 mg Zn/kg, but provided hay with a higher Zn concentration, in order to maintain feed intake.

Zinc dependent enzymes

Zinc metalloenzymes, such as alkaline phosphatase (ALP), in plasma or serum, erythrocytes, erythrocyte membranes and other specific cell types have been studied in humans as possible biomarkers for Zn status.

Alkaline phosphatase is a Zn-containing enzyme found in blood, bone and other tissues and is involved in the formation of hydroxyapatite (Peters, 2006). Hydroxyapatite, an inorganic calcium-containing mineral found in normal bone and teeth, lends rigidity to these structures. The ALP activity is Zn-dependent, as Zn is present in the active centre of the enzyme (Fisher, 1975; Galdes and Hill, 1979; Coleman, 1992; King, 2011). Alkaline phosphatase is most frequently determined in plasma or serum.

Humans and laboratory animals

In humans, the response of plasma/serum ALP to dietary Zn intake levels varies greatly. Some studies reported no significant changes during depletion and repletion (Ruz *et al.,* 1991), whereas other studies reported a decreased plasma/serum ALP concentration during depletion and increased concentration during repletion by Zn addition to the diet (Delves, 1985; Lowe *et al.*, 2004). After repletion, plasma ALP concentrations responded similarly to plasma Zn concentration, although more slowly, and they returned later to values higher than baseline (Lowe *et al.,* 2004). Generally, ALP activity is more reduced during severe Zn deficiency compared to moderate Zn deficiency (Gibson *et al.*, 2008).

Production animals

In contrast to human studies, literature regarding the plasma/serum ALP response to low dietary Zn intakes in production animals is very limited. The reported responses should therefore be interpreted with caution.

Piglets and calves seem to have a lower plasma ALP concentration at a low dietary Zn concentration (non-supplemented). In one study, however, ALP concentration only responded at specific sampling times in lambs and heifers (Spears, 1989) (Table 3.1-3.3). The plasma ALP response to an increase in dietary Zn concentrations varies between studies. Most studies observed an increase in plasma ALP concentration in piglets and lambs, with a plateau observed around 30-53 mg added Zn/kg diet for piglets (Spears and Hansen, 2008; Bikker and Jongbloed, 2014). Other studies in calves and broilers reported a lack of response in plasma ALP concentrations to increased dietary Zn concentrations (Table 3.1-3.4).

In cows, no responses in plasma ALP concentrations have been reported (Table 3.1). In ewes and sows, during low dietary Zn intake, plasma ALP concentrations seem to decrease except for one study in sows in which no effect was found (Kalinowski and Chavez, 1984) (Table 3.2 and 3.3). These differences are probably due to the duration of the study period and parity; in the latter study, multiparous sows (parity 4-7) were provided the low Zn diet from the last 4 weeks of gestation until the first 2 weeks of lactation, whereas other studies used primiparous sows and observed the animals for a longer period. With increasing dietary Zn concentrations, plasma ALP responses are even more variable, presumably depending on reproductive phase and Zn supplementation level. Higher plasma ALP concentrations were only found in sows when dietary Zn concentration reached pharmacological (>500 mg added Zn/kg) levels (Hill *et al.,* 1983a,b) (Table 3.3).

Suitability of plasma ALP concentration for zinc status assessment

In both humans and production animals, plasma ALP responses vary greatly between studies, which hamper its use for Zn status assessment. Plasma ALP is not suitable to determine Zn requirements in ruminants, as it may respond only at certain sampling times within an experiment. Spears and Hansen (2008) reported that the use of plasma ALP in ruminants is suitable to determine Zn bioavailability. However, this seems to be non-applicable based on previous studies (Spears, 1989; Kessler *et al.,* 2003). Indeed, calves seem to have a low plasma ALP concentration at low dietary Zn intake (non-supplemented), but it did not respond to increased dietary Zn concentrations while other biomarkers did. A similar phenomenon is observed in cows. In piglets, plasma ALP seems to respond more to both low and high dietary Zn intake, thus plasma ALP seems sensitive to dietary Zn intake. The use of plasma ALP to determine Zn requirements is questionable, because the plateau found in some studies is lower than plasma ALP concentration found in control (non-supplemented) versus Zn supplemented piglets in other studies. A similar suggestion appears in Spears and Hansen (2008) for pigs, (*i.e.* that it is unclear whether plasma ALP is suitable to determine Zn bioavailability) because some studies in piglets found no effect of Zn source on plasma ALP concentration (Revy *et al.,* 2002 and 2004). For broilers, plasma ALP is likely not suitable for Zn status assessment, but this requires further research that also includes laying hens.

Table 3.3. Responses of zinc status biomarkers in pigs fed varying dietary zinc concentrations[*].

Category	Basal diet (mg Zn/kg)	Intervention (added Zn)	Responses to Zn addition[†]											References
			Body tissues						Blood			Excretion		
			Liver	Pancreas	Kidney	Intestine	Muscle	Bone	Zn	ALP	MT	Faeces	Urine	
Piglets	30	0, 15, 30, 45, 60, 100	+ < 45					+	+ < 60	+ < 30				Bikker *et al.*, 2011
Piglets	100-125	0, 150, 300, 450, 2000							+ > 2000			+		Buff *et al.*, 2005
Piglets[‡]	165	0 - 2000							+ > 2000			+ > 2000		Carlson *et al.*, 2004
Piglets	100	0, 100, 250, 1000, 2500				+ musosa Zn			+	=				Carlson *et al.*, 2007
Piglets[§]	52-84	25, 100/150							+	+		+		Creech *et al.*, 2004
Piglets	12	0, 78	+	+	=	=		+	+	+				Prasad *et al.*, 1969
Piglets[¶]	28	0, 10, 20, 30	+ > 20					+ > 20	+	+				Revy *et al.*, 2002
Piglets	32	0, 20	=					+	+ no phytase added	+ no phytase added		+	=	Revy *et al.*, 2004
Piglets	33	0, 10, 25, 40, 60, 80	+					+	+ < 59	+ < 53				Revy *et al.*, 2006
Piglets	25/38	0, 15						+	+ depending Zn source	+				Schlegel *et al.*, 2010
Fattening[‡]	27-32	0, 5, 10, 20, 40, 80						+ < 20	+ < 20					Wedekind *et al.*, 1994

Table 3.3. *Continued*

Category	Basal diet (mg Zn/kg)	Intervention (added Zn)	Responses to Zn addition[†]											References
			Body tissues						Blood			Excretion		
			Liver	Pancreas	Kidney	Intestine	Muscle	Bone	Zn	ALP	MT	Faeces	Urine	
Sows	33	0, 50							+					Hedges *et al.*, 1976
Sows	35	0, 50, 500, 5000	+	+ > 5000	+ > 5000		=		+ > 5000	+ > 5000				Hill *et al.*, 1983a,b
Sows	32	0, 100	+					+	+	+				Hoekstra *et al.*, 1967
Sows	13	0, 50							+ during lactation	=				Kalinowski and Chavez, 1984
Sows	10	0, 40							+	+			+ [Zn] & excretion = volume	Kalinowski and Chavez, 1986
Sows	10	0, 40	+		=	+	=	+						Kalinowski and Chavez, 1991
Sows[‡]	72-100	72-168	+ 12-35 mo, not at 8 mo											Peters, 2006
Sows[‡]	72-100	72-168	+											Peters *et al.*, 2010

Zn, plasma Zn; ALP, plasma Alkaline phosphatase; MT, Metallothionein; [Zn], Zn concentration; mo, months of age.

[*] Responses to Zn addition in the value of the observed biomarker are indicated as no effect (=), a decrease (-), or an increase (+), until (<) or beyond (>) a specific Zn addition.

† Responses are represented related to the added Zn concentration to the diet and not total dietary Zn concentration.
‡ Studies consisted of more than 1 trial. Carlson *et al.* (2004): basal diet, 165 mg Zn/kg. Added Zn concentration trial 1, 2 and 3: T1= 0, 125, 250, 375, 500, 2000 mg Zn/kg, T2= 0, 50, 100, 200, 400, 800, 2000 mg Zn/kg, T3= 0, 200, 400, 2000 mg Zn/kg. Wedekind *et al.* (1994): basal diet during growing phase: 32 mg Zn/kg, finishing phase: 27 mg Zn/kg. Added Zn concentration trial 1 and 2: T1= 0, 5, 10, 20, 40, 80 mg Zn/kg, T2= 0, 7.5 and 15 mg Zn/kg from different sources. Peters (2006) and Peters *et al.* (2010): basal diet varied between 72 and 100 mg Zn/kg among gestation (parity 1-2 and parity 3-6) and lactation diets and between Zn sources used. Added Zn concentration gestation 1 & 2: 73,75 (inorganic Zn source) and 86,118 (organic Zn source) mg/kg Zn, gestation 3-6: 72,73 (inorganic Zn source) and 111,168 (organic Zn source) mg Zn/kg, lactation 1-6: 73,124 (inorganic Zn source) and 85,95 (organic Zn source) mg Zn/kg.
§ Studies used a Zn supplemented control diet (at or above Zn requirements) and lower Zn supplementation levels as treatment group(s). Creech *et al.* (2004): basal diet Zn concentration nursery 84 mg/kg, growing 67-78 mg/kg, reduced supplemented 52-71 mg/kg. Added Zn concentration nursery 150 mg/kg, growing 100 mg/kg, reduced supplemented 25 mg/kg
¶ Plasma Zn and plasma ALP concentration increased linearly and quadratically, although the quadratic response only explained 6% and 12% of the total variance, respectively.

Transport proteins

Metallothionein is a small cysteine-rich protein involved in the transfer and sequestering of absorbed metal ions, particularly when the influx of metal ions is high, as well as in cellular detoxification (Underwood and Suttle, 1999; McDowell, 2003; King, 2011). It is also involved in holding Zn in the appropriate valance state or orientation for absorption (Hill and Link, 2009). Metallothionein is produced by the liver and can strongly bind to Zn. As such, it is a major storage form of Zn. It is particularly concentrated in the liver, kidneys, pancreas and intestinal mucosa (McDowell, 2003; Gibson *et al.*, 2008; King, 2011) and also present in serum at a low concentration (King, 1990; Suttle, 2010; Roohani *et al.*, 2013). Metallothionein thus has a central role in Zn homeostasis. Responses of matrices of MT described in literature are body tissues and MT in plasma, erythrocytes, leukocytes, urine and mRNA.

Humans and laboratory animals

In humans, MT in serum, erythrocyte and body tissues respond to changes in dietary Zn intake (King, 1990 and 2011; Gibson *et al.*, 2008; Lowe *et al.*, 2009; Roohani *et al.*, 2013). Especially the circulating MT concentration appears to correlate with dietary Zn intake (Lowe *et al.*, 2009; Roohani *et al.*, 2013).

The MT concentration in serum and erythrocyte decreased during moderate and severe dietary Zn deficiency and rose again during the repletion period (Grider *et al.*, 1990; King, 1990; Gibson *et al.*, 2008). Increased dietary Zn concentration (Zn supplementation) resulted in a significantly greater MT concentration in erythrocytes (King, 1990; Sullivan *et al.* 1998; Cao and Cousins, 2000). For serum MT concentration, no studies used this biomarker to explore possible effects of Zn supplementation.

The response of MT in body tissues during moderate and severe dietary Zn deficiency is unclear (King, 2011) due to the lack of studies performed. Metallothionein concentration in body tissues increased during dietary Zn supplementation (above maintenance requirements) (King, 1990), thereby increasing the levels of MT at the apical side of the intestinal lumen together with the major fraction of Zn in free-Zn form and minor changes in MT levels at the basolateral side, which facilitates Zn transport in intestinal cells (Moltedo *et al.*, 2000).

Production animals

To our knowledge, literature focussing on MT responses to dietary Zn concentration in production animals is lacking, especially for erythrocyte and serum concentration. In calves and piglets, liver MT concentrations seem to increase only at pharmacological dietary Zn concentrations (calves: 500

mg added Zn/kg DM, piglets: 3000 mg added Zn/kg) (Table 3.1 and 3.3, piglets: Carlson *et al.*, 1999), whereas for broilers, (pancreas) MT concentrations increased with increasing dietary Zn concentrations, reaching a plateau at 100- 120 mg added Zn/kg diet (Table 3.4).

Suitability of metallothionein concentration for zinc status assessment

Although MT concentration, especially circulating MT, appears to correlate with dietary Zn intake in humans, this has not yet been confirmed in production animals. Nevertheless, MT could be a potential biomarker for Zn status assessment, based on the function of MT in Zn metabolism and in maintaining Zn homeostasis, especially when serum MT is used together with plasma Zn. Using both biomarkers, a differentiation between suboptimal Zn intake (low plasma Zn and serum MT concentrations) and redistribution of tissue Zn (low plasma Zn and high serum MT concentrations) may be distinguished (King, 1990 and 2011; Gibson *et al.*, 2008). Further research is required to test this postulation in production animals.

Faecal and urinary zinc excretion

Faecal Zn excretion is the major excretion route for Zn; urinary Zn is a minor route. Zinc concentration in the faeces consists of unabsorbed dietary Zn and endogenous Zn losses. The quantity of Zn absorbed in the small intestine influences the amount of Zn in endogenous tissue pools, which in turn may be associated with the fraction of endogenous Zn excreted (King, 1990).

Humans and laboratory animals

During low dietary Zn intake, faecal Zn concentration decreases (Jackson *et al.*, 1982; King, 1990; Hess *et al.*, 2007) and during periods of high dietary Zn intake, the endogenous faecal Zn excretion is increased (King, 1990) as adjustments to maintain homeostasis.

Renal losses remained constant over a wide range of dietary Zn intakes but declined quickly when the dietary Zn intake was far below the requirements (King *et al.*, 2000; King, 2011). Urinary Zn excretion is possibly mediated by an adjustment in renal tubular Zn transport (King *et al.*, 2000).

Production animals

During low dietary Zn intake, a decreased faecal Zn concentration was found in piglets (Table 3.3), whereas the faecal Zn excretion in piglets and broilers increased linearly with increased dietary Zn concentration (Table 3.3 and 3.4). The urinary Zn concentration seems unresponsive to increased dietary Zn concentrations, although it increased in piglets fed pharmacological dietary Zn levels (3000 mg added Zn/kg compared to 150 or 500 mg added Zn/kg diet) (Case and Carlson, 2002),

indicating that urinary Zn only fluctuates during extremely high dietary Zn concentration (similar to in humans).

Dairy cattle lactating for the first time had a decreased faecal Zn excretion and increased Zn absorption during low dietary Zn intake (Neathery *et al.*, 1973) (Table 3.1). In sows, a decreased urinary Zn excretion was also found (Kalinowski and Chavez, 1986). Available literature for the faecal and urinary response to increased dietary Zn concentrations in breeding production animals is very limited; only in dairy goats was an increased faecal Zn excretion found (Table 3.2).

Suitability of faecal and urinary zinc excretion for zinc status assessment

In humans and production animals, Zn is mainly excreted via the faeces; this excretion depends more on dietary Zn intake than the urinary Zn excretion route. Faecal Zn excretion is probably regulated in accordance with changes in recent Zn absorption and Zn status (Hambidge, 2003). However, the suitability of faecal Zn excretion to determine Zn requirements is questionable and is hampered by the limited number of available studies. No plateau is reported in production animals, because small changes in faecal Zn excretion may not be detectable until dietary Zn concentration rises above Zn requirements. In the presence of excessive Zn, unabsorbed dietary Zn will be excreted as the major adjustment strategy to maintain Zn homeostasis.

Faecal Zn excretion also seems to be a less suitable biomarker for Zn bioavailability assessment. Studies in piglets did not find a response dependent on Zn source used (Revy *et al.*, 2002, 2004), suggesting again that faecal Zn excretion is not accurate to detect small improvements in Zn absorption compared with plasma Zn and body tissues, which show higher sensitivity.

Table 3.4. Responses of zinc status biomarkers in poultry fed varying dietary zinc concentrations[*].

Category	Basal diet (mg Zn/kg)	Intervention (added Zn)	Responses to Zn addition[†]											References
			Body tissues							Blood		Excretion		
			Liver	Pancreas	Kidney	Intestine	Muscle	Bone	Zn	ALP	MT	Faeces	Urine	
Broilers	20.4	0, 20, 40, 50, 80	- > 0					+ < 40	=			+		Bao *et al.*, 2007
Broilers	34	0, 34, 147	+						=					Bartlett and Smith, 2003
Broilers	28.4	0, 20, 40, 60, 80, 100, 120, 140		+ < 40	=			+ < 60-80			+ < 100-120 pMT			Huang *et al.*, 2007
Broilers	27.7	0, 20, 40, 60, 80, 100, 120, 140		+				+		=	+ pMT			Liao *et al.*, 2012
Broilers[‡]	20-25	0 - 179						+ < 40	+ < 40	=		+		Mohanna and Nys, 1999
Broilers	25/38	0, 15						+	+	=				Schlegel *et al.*, 2010
Broilers	29	0, 10, 20, 40, 80, 160, 320	+		+			+ < 40						Sunder *et al.*, 2008
Broilers	24	0, 30, 60, 90	Highest for 90 mg Zn/kg + 200 FTU/kg							=				Zaghari *et al.*, 2015

Table 3.4. *Continued*

Category	Basal diet (mg Zn/kg)	Intervention (added Zn)	Responses to Zn addition†											References
			Body tissues						Blood			Excretion		
			Liver	Pancreas	Kidney	Intestine	Muscle	Bone	Zn	ALP	MT	Faeces	Urine	
Laying hens	46	0, 25, 50, 100, 200							0 & 100 mg Zn/kg higher than other Zn suppl. levels					Kaya *et al.*, 2001
Laying hens	35	0, 20 g/kg	+ until d3-4	+ until d10	+ until d3-4									Williams *et al.*, 1989

Zn, plasma Zn; ALP, plasma Alkaline phosphatase; MT, Metallothionein; pMT, pancreas MT concentration; Suppl. levels, supplementation levels

* Responses to Zn addition in the value of the observed biomarker are indicated as no effect (=), a decrease (-), or an increase (+), until (<) or beyond (>) a specific Zn addition.

† Responses are represented related to the added Zn concentration to the diet and not total dietary Zn concentration.

‡ Studies consisted of more than 1 trial. Mohanna and Nys (1999): basal diet trial 1 and 2: T1= 20 mg Zn/kg, T2= 25 mg Zn/kg. Added Zn concentration T1= 0, 10, 20, 40 mg Zn/kg, T2= 0, 40, 50, 66, 85, 179 mg Zn/kg.

Functional indicators

This group of indicators reflects the functional consequences of Zn deficiency using rapid and mostly non-invasive methods (de Benoist *et al.,* 2007; Gibson *et al.,* 2008), such as growth rate, developmental outcome and presence of infectious diseases (Fischer Walker and Black, 2007; de Benoist *et al.,* 2007; Gibson *et al.,* 2008; Roohani *et al.,* 2013). Generally, these indicators have a low specificity and are associated with other nutritional deficiencies or infection (de Benoist *et al.,* 2007). Therefore, functional indicators should be evaluated in response to Zn supplementation in controlled experimental designs (King, 1990; Gibson *et al.,* 2008).

Developmental outcome and presence of infectious have only been scarcely addressed in studies in production animals (*e.g.* prevalence of diarrhoea), whereas growth, feed intake and feed efficiency are more comprehensively reported in production animals. Within this context, the impact of dietary Zn concentration on milk and egg production and on reproductive performance in production animals is also addressed.

Prevalence of diarrhoea

Humans and laboratory animals

Acute and persistent diarrhoea is a condition associated with Zn deficiency in humans. Zinc deficiency can be a cause or consequence of diarrhoea (Hambidge, 1992). As a causal factor, a mild Zn deficiency may contribute to the duration and severity of acute diarrhoea (Hambidge, 1992). Severe Zn deficiency results in acrodermatitis enteropathica with diarrhoea as one of the main symptoms (Hambidge, 1992). During diarrhoea, excessive faecal Zn losses are reported along with decreased plasma and tissue Zn concentrations, contributing to the development of Zn deficiency (Hambidge, 1992; Hambidge and Krebs, 2007).

An increased dietary Zn intake showed beneficial effects on the prevention and treatment of acute and persistent diarrhoea (Hambidge, 1992; Hambidge and Krebs, 2007; Fischer Walker and Black, 2007).

Production animals

In piglets, pharmacological dietary Zn concentrations are fed to prevent post-weaning diarrhoea for two weeks that substitutes antibiotics (Poulsen, 1995; Peters, 2006; Bikker and Jongbloed, 2014). Some studies have taken the associated faecal colour and faecal consistency into account, which improved at pharmacological dietary Zn concentrations (Poulsen, 1995; Peters, 2006). Lower Zn

supplementation levels (between 15 to 100 mg added Zn/kg) may not affect faecal consistency (Bikker *et al.*, 2011).

Suitability of prevalence of diarrhoea for zinc status assessment

Despite a decreased incidence and prevalence of diarrhoea during Zn supplementation in humans (Fischer Walker and Black, 2007) this functional indicator has a low specificity and is difficult to assess in a controlled experimental design (de Benoist *et al.,* 2007). The suitability of this functional indicator seems negligible for production animals, because solely pharmacological dietary Zn concentrations are provided to weaned piglets.

Growth, feed intake and feed efficiency

Humans and laboratory animals

Growth retardation is an early response to Zn deficiency in humans and laboratory animals to maintain whole body Zn concentrations, thereby redistributing Zn in tissues and fluids in which Zn concentration may increase or decrease (King, 1990).

In 21 randomised studies with children from birth to 17 years and in a meta-analysis of pre-pubertal children, longitudinal growth was positively affected during dietary Zn supplementation, whereas in seven randomised studies, no effect was found (Brown *et al.,* 2002; Fischer Walker and Black, 2007; Gibson *et al.*, 2008).

In humans, the length-for-age is preferably used over weight-for-age as a functional outcome associated with the risk of Zn deficiency in populations (de Benoist *et al.,* 2007; Gibson *et al.,* 2008; Roohani *et al.,* 2013), because length-for-age represent linear growth as the primary response to increased dietary Zn intake, whereas weight gain is likely an indirect measurement of increased linear growth (de Benoist *et al.,*, 2007; Gibson *et al.,* 2008).

For determination of Zn requirements, studies showed that the physical growth rate (*e.g.* linear growth and weight gain) was a useful response criterion in children during dietary Zn supplementation, but not in adults (Naithani *et al.,* 2014).

Production animals

While the length-for-age is preferably used over weight-for-age in children, this indicator is less suited for production animals in which growth performance is expressed as weight gain, weight changes or average daily gain (ADG).

Growth performance and/or feed efficiency was reduced in calves and lambs at low dietary Zn intake and weight gain ceased completely if a diet without supplemental Zn was fed (Mills *et al.*, 1967) (Table 3.5). The majority of the studies performed in growing production animals were related to the effect of increased dietary Zn concentrations; for cattle and pigs, however, the results described in some extensive reports (cattle: Spears and Weiss, 2014, pigs: Jongbloed *et al.,* 2004; Bikker and Jongbloed, 2014) are inconsistent. Some studies found positive effects on growth performance in heifers, growing steers and lambs during the first 56 days of the experiment and in goats when the dietary Zn concentration was sufficiently high (Table 3.5). However, other studies in calves, heifers, fattening bulls and lambs found no effect of increased dietary Zn concentrations on growth performance, feed intake and/or feed efficiency (Table 3.5) and for cattle, reported in Spears and Weiss (2014). Positive effects in piglets and broilers were also found, reaching in some studies a plateau at 15 mg added Zn/kg for piglets (Revy *et al.,* 2006; Bikker *et al.,* 2011) and a plateau up to 25- 40 mg added Zn/kg for broilers (Table 3.6). In contrast, other studies found no effect of increased dietary Zn concentrations (Table 3.6, Bikker and Jongbloed, 2014).

In breeding production animals, weight changes, feed intake (although adequately monitored) and feed efficiency are rarely addressed. During low dietary Zn intake in ewes, some studies found a decreased feed intake and feed efficiency at week 20 of gestation and after parturition (Table 3.5). However, in lactating cows and sows, neither weight changes nor a reduced feed intake were observed during low (non-supplemented) dietary Zn intake (Tables 3.5 and 3.6). Weight changes and feed intake improvements were found with increased dietary Zn concentration in goats and laying hens, although the laying hens were reared under low ambient temperatures (Tables 3.5 and 3.6). No effect of Zn supplementation on bodyweight was found in other studies on cows, ewes, sows and laying hens (Tables 3.5 and 3.6).

Suitability of growth performance for zinc status assessment

Growth performance is used in humans and production animals as a functional indicator for Zn status assessment. However, in growing production animals, contradictory results regarding its response to dietary Zn intake is found. In piglets and broilers, an increase in weight gain with increasing Zn supply and a plateau for maximal growth rate was reported in some studies (Jongbloed *et al.,* 2004). This suggests that growth performance is a sensitive indicator to determine Zn requirements if studies are performed under controlled experimental conditions, otherwise other factors besides dietary Zn also influence growth (low specificity). The sensitivity of growth

performance is also suitable to determine Zn bioavailability in studies using dietary Zn concentrations below Zn requirements (Jongbloed *et al.*, 2004; Spears and Hansen, 2008).

In mature human subjects and breeding production animals, weight changes seem unsuitable as functional indicator for Zn status assessment, as the main function of Zn is growth and development.

Table 3.5. Responses of functional zinc status indicators in ruminants fed varying dietary zinc concentrations[*].

Species	Basal diet (mg Zn/kg DM)	Intervention (added Zn)	Responses to Zn addition[†]							Reference
			Feed intake	Feed efficiency	Growth performance	Milk production			Reproduction	
						Yield	Composition	SCC		
Cattle										
Calves	60	0, 150, 300	=		=					Kincaid *et al.*, 1997
Calves	33	0, 200, 600								Miller *et al.*, 1970
Calves	1.2	0, 2, 8, 25			+ at 8					Mills *et al.*, 1967
Calves	20	0, 200	=		=					Rojas *et al.*, 1996
Calves	28	0, 20, 500	=	=	=					Wright and Spears, 2004
Heifers	23.8	0, 25	=	+ first 56 d	+ first 56 d					Spears, 1989
Steers[‡]	26	0, 81			+					Brazle, 1993
Bulls[‡]	-	0, 150	=	-	-					Fagari-Nobijari *et al.*, 2012
Bulls	35	0, 10	=	=	=					Kessler *et al.*, 2003
Cows	154.8 mg Zn/cow	0, 910.2 mg/cow				+				Anton *et al.*, 2013
Cows[‡]	-	-				+				Enjalbert *et al.*, 2006
Cows[‡]	35.5-53.6	0				=	=	=	=	Formigoni *et al.*, 2011
Cows	31	0, 6.1, 18.3			=					Lethbridge, 2009
Cows	16.6	0, 22.9					= Milk Zn			Neathery *et al.*, 1973
Cows	37.6	0, 40	=				=			Wang *et al.*, 2013
Cows	36	0, 60, 300	=		=	+				Cope *et al.*, 2009
Goats										
1-y-old	22.3	0, 15, 30, 45	+ > 30, 45	+ > 30, 45						Jia *et al.*, 2008
1-y-old	22	0, 40, 120, 200	=		+					Puchala *et al.*, 1999
Dairy	78	0, 1 g/d	Tended to +			=				Salama *et al.*, 2003

Table 3.5. *Continued*

Species	Basal diet (mg Zn/kg DM)	Intervention (added Zn)	Responses to Zn addition[†]							Reference
			Feed intake	Feed efficiency	Growth performance	Milk production			Reproduction	
						Yield	Composition	SCC		
Sheep										
Lambs	1.2	0, 2, 7.1, 24			+ at 7					Mills *et al.*, 1967
Lambs	35	0, 20, 40	=							Pal *et al.*, 2014
Lambs	2.8	0, 5 (d0-42), 15 (d42-56)	=	+	+					Spears, 1989
Lambs	4	0, 10, 17, 29	+ > 10		+ > 10					White *et al.*, 1994
Ewes[§]	<1 mg/kg + 16-20 mg/kg	0, 20					+ Colostrum Zn		+	Apgar and Fitzgerald, 1987
Ewes[§]	<1 mg/kg + 15-20 µg/g DM	0, 100	+ wk 20 gestation		+ after lambing				=	Apgar and Travis, 1979
Ewes	15	0, 15, 30	=		=	=	+ Milk Zn			Zali and Ganjkhanlou, 2009

Growth performance, incudes results of growth, average daily gain (ADG), and bodyweight (BW) changes; SCC, somatic cell count; DM, dry matter basis.

[*] Responses to Zn addition in the value of the observed biomarker are indicated as no effect (=), a decrease (-), or an increase (+), until (<) or beyond (>) a specific Zn addition.

[†] Responses are represented related to the added Zn concentration to the diet and not total dietary Zn concentration.

[‡] Studies with varying dietary Zn concentration during gestation and lactation or unknown Zn interventions. Formigoni *et al.* (2011): basal diet, 35.5 mg Zn/kg DM during gestation and 53.6 mg Zn/kg DM during lactation. No Zn addition, but different Zn source, 35.5 mg Zn/kg DM during gestation or 51.3 Zn/kg DM during lactation. The retrospective study of Enjalbert *et al.* (2006) used plasma Zn concentrations from 2080 cattle herds to evaluate relationships between Zn status and (re)production characteristics, but did not include dietary Zn concentrations.

[§] Studies provided a semipurified diet with <1 mg Zn/kg, but provided hay with a higher Zn concentration, in order to maintain feed intake.

Table 3.6. Responses of functional zinc status indicators in monogastric production animals fed varying dietary zinc concentrations[*].

Species	Basal diet (mg Zn/kg)	Intervention (added Zn)	Responses to Zn addition[†]						Reference
			Feed intake	Feed efficiency	Growth performance	Milk/egg production		Reproduction	
						Yield	Composition		
Pigs									
Piglets	30	0, 15, 30, 45, 60, 100	+ < 15	+ < 15	+ < 15				Bikker *et al.*, 2011
Piglets	100-125	0, 150, 300, 450, 2000	=	+ > 2000	+ > 2000				Buff *et al.*, 2005
Piglets[‡]	165	0 - 2000	=	=	+ > 2000				Carlson *et al.*, 2004
Piglets[§]	52-84	25, 100/150	=	=	=				Creech *et al.*, 2004
Piglets	12	0, 78		+	+				Prasad *et al.*, 1969
Piglets	28	0, 10, 20, 30	=	=	=				Revy *et al.*, 2002
Piglets	32	0, 20		+	+				Revy *et al.*, 2004
Piglets	33	0, 10, 25, 40, 60, 80	= except 10 (-)						Revy *et al.*, 2006
Piglets	25/38	0, 15	=	=	=				Schlegel *et al.*, 2010
Fattening[‡]	27-32	0, 5, 10, 20, 40, 80	=	=	=				Wedekind *et al.*, 1994
Sows	33	0, 50						=	Hedges *et al.*, 1976
Sows	35	0, 50, 500, 5000		=	= except 5000 at slaughter (-)			+ abnormal piglets (n)/ litter	Hill *et al.*, 1983a,b
Sows	32	0, 100			=			+	Hoekstra *et al.*, 1967
Sows	13	0, 50	=		=		= Colostrum & milk Zn	=	Kalinowski and Chavez, 1984
Sows	10	0, 40	=		=		+ Milk Zn	+	Kalinowski and Chavez, 1986

Table 3.6. *Continued*

Species	Basal diet (mg Zn/kg)	Intervention (added Zn)	Responses to Zn addition†						Reference
			Feed intake	Feed efficiency	Growth performance	Milk/egg production		Reproduction	
						Yield	Composition		
Sows‡	72-100	72-168	=	=	=			+	Peters and Mahan, 2008
Sows‡	72-100	72-168			=		- Colostrum Zn		Peters *et al.*, 2010
Sows	35	0, 50					=	=	Pond and Jones, 1964
Poultry									
Broilers	20.4	0, 20, 40, 50, 80	+ < 20	+ < 40	+ < 40				Bao *et al.*, 2007
Broilers	34	0, 34, 147	=	=	=				Bartlett and Smith, 2003
Broilers	28.4	0, 20, 40, 60, 80, 100, 120, 140	+ < 20	=	+ < 20				Huang *et al.*, 2007
Broilers	27.7	0, 20, 40, 60, 80, 100, 120, 140			=				Liao *et al.*, 2012
Broilers‡	20-25	0 - 179	+ < 25	+ < 10	+ < 25				Mohanna and Nys, 1999
Broilers	25/38	0, 15	=	=	=				Schlegel *et al.*, 2010
Broilers	29	0, 10, 20, 40, 80, 160, 320	=	=	=				Sunder *et al.*, 2008
Broilers	24	0, 30, 60, 90	=	=	+ at 30				Zaghari *et al.*, 2015
Laying hens	46	0, 25, 50, 100, 200	=	=	=	=	Yolk Zn 50 mg Zn/kg higher than other suppl. levels		Kaya *et al.*, 2001

Table 3.6. *Continued*

Species	Basal diet (mg Zn/kg)	Intervention (added Zn)	Responses to Zn addition†					Reference
			Feed intake	Feed efficiency	Growth performance	Milk/egg production		Reproduction	
						Yield	Composition		
Laying hens	35	0, 20 g/kg	- at d4		- at d4		+ until d3-4 for egg yolk Zn		Williams *et al.*, 1989

Growth performance, incudes results of growth, average daily gain (ADG), and bodyweight (BW) changes; SCC, somatic cell count; Suppl. levels, supplementation levels.

* Responses to Zn addition in the value of the observed biomarker are indicated as no effect (=), a decrease (-), or an increase (+), until (<) or beyond (>) a specific Zn addition.

† Responses are represented related to the added Zn concentration to the diet and not total dietary Zn concentration.

‡ Studies consisted of more than 1 trial. Carlson *et al.* (2004): basal diet, 165 mg Zn/kg. Added Zn concentration trial 1, 2 and 3: T1= 0, 125, 250, 375, 500, 2000 mg Zn/kg, T2= 0, 50, 100, 200, 400, 800, 2000 mg Zn/kg, T3= 0, 200, 400, 2000 mg Zn/kg. Wedekind *et al.* (1994): basal diet during growing phase: 32 mg Zn/kg, finishing phase: 27 mg Zn/kg. Added Zn concentration trial 1 and 2: T1= 0, 5, 10, 20, 40, 80 mg Zn/kg, T2= 0, 7.5 and 15 mg Zn/kg from different sources. Peters (2006) and Peters *et al.* (2010): basal diet varied between 72 and 100 mg Zn/kg among gestation (parity 1-2 and parity 3-6) and lactation diets and between Zn sources used. Added Zn concentration gestation 1 & 2: 73,75 (inorganic Zn source) and 86,118 (organic Zn source) mg/kg Zn, gestation 3-6: 72,73 (inorganic Zn source) and 111,168 (organic Zn source) mg Zn/kg, lactation 1-6: 73,124 (inorganic Zn source) and 85,95 (organic Zn source) mg Zn/kg. Mohanna and Nys (1999): basal diet trial 1 and 2: T1= 20 mg Zn/kg, T2= 25 mg Zn/kg. Added Zn concentration T1= 0, 10, 20, 40 mg Zn/kg, T2= 0, 40, 50, 66, 85, 179 mg Zn/kg.

§ Studies used a Zn supplemented control diet (at or above Zn requirements) and lower Zn supplementation levels as treatment group(s). Creech *et al.* (2004): basal diet Zn concentration nursery 84 mg/kg, growing 67-78 mg/kg, reduced supplemented 52-71 mg/kg. Added Zn concentration nursery 150 mg/kg, growing 100 mg/kg, reduced supplemented 25 mg/kg.

Milk and egg production

Production animals

During a period of low dietary Zn intake, reduced milk production was found in dairy cattle, as well as reduced milk Zn concentration in ewes and sows. However, one study in sows reported no reduction in Zn concentration in colostrum and milk after reduced dietary Zn intake (Tables 3.5 and 3.6). Some studies reported an increase in milk production and an altered milk composition in dairy cows with increased dietary Zn concentrations, but others did not. The colostrum and milk Zn concentration was increased in cows, ewes and sows given Zn supplemented feed (Table 3.5 and 3.6; Spears and Weiss, 2014). Increased Zn concentrations were especially found in the cream but not the skimmed milk fraction (Hosnedlova *et al.,* 2007).

To the best of our knowledge, the effect of insufficient dietary Zn concentrations on egg production in laying hens has not been studied. At increased dietary Zn concentration, most studies observed no effects (Table 3.6). Based on one study, it seems that the Zn concentration in egg yolk linearly increased with increasing dietary Zn concentrations after 3-4 days (Williams *et al.,* 1989). However, another study in laying hens found no linear increase for yolk Zn concentration; an addition of 50 mg added Zn/kg compared to other supplemental concentrations (0, 25, 100 and 200 mg added Zn/kg) showed the highest yolk Zn concentration (Kaya *et al.,* 2001).

Usefulness of milk and egg production for zinc status assessment

The production of milk and eggs did not consistently respond to dietary Zn intake, although only a limited number of studies have been done on laying hens. Not only the response of milk yield and composition to dietary Zn supply but also colostrum and milk Zn concentration and egg production and yolk Zn concentration show variable responses. Responses differ between studies and depend on species and other factors. This results in a lower sensitivity and hampers the use of milk and egg production as functional indicator for Zn status assessment.

Reproductive performance

Production animals

Reproductive performance in production animals (males and females) is impaired during low dietary Zn intake (Miller, 1979; Hosnedlova *et al.,* 2007; Suttle, 2010; Kumar *et al.,* 2011). In males, retarded testicle development, impaired spermatogenesis and poor sperm quality was found. In females, impaired synthesis and secretion of LH and FSH, abnormal ovary development, abnormal oestrus cycle, difficulties in becoming gravid, reduced number of fertile eggs, extended gestation period, prolonged parturition, retained placentas, low viability of their progeny and

abortions were observed. Some species-specific effects and variations between studies may be present.

Increased dietary Zn concentrations do not seem to improve reproductive performances in females receiving a low Zn diet as preliminarily based on a limited number of studies in cows and ewes (Table 3.5). In sows, Zn inclusion in the diet seems to improve reproductive performances, but not in all studies (Table 3.6). This may be related to differences in study design (*e.g.* age of sows, reproductive capacity, duration of Zn supplementation period, high dietary Ca concentrations and response parameters) rather than dietary Zn concentration which was within a similar range. The reproductive performance of males during increased dietary Zn concentrations has not been studied, except for one study in boars (11-24 months of age) where sperm production but not sperm quality increased with increased dietary Zn concentration (Liao *et al.,* 1985).

Usefulness of reproductive performance for zinc status assessment

For reproduction, low dietary Zn intake seems to have a larger effect than increased dietary Zn concentrations. This makes reproductive performance more suitable for diagnosing Zn deficiency than for determining Zn requirements, because responses are not sensitive to dietary Zn intake, at least not in females and based on a limited number of studies. Still, other factors then dietary Zn concentration may cause a declined reproductive performance, lowering its specificity. Potentially, reproduction may be promising as functional indicator to determine Zn bioavailability in cows, based on a meta-analysis of Rabiee *et al.* (2010) that encompasses 20 studies. However, other studies did not find an association in reproductive performance with trace mineral source in both males and females (Althouse *et al.,* 2000; Karkoodi *et al.,* 2012). More research is required.

Conclusion

Several biomarkers for Zn status assessment that have been described in humans have also been evaluated for suitability in growing and breeding production animals. Different responses of the biomarkers, especially those of body tissues, were found between humans and production animals and among animal species.

In general, dietary Zn intake can be used as additional biomarker. Body tissues, except for bone in poultry, are less suitable compared to plasma Zn concentration. Plasma ALP concentration does not seem sensitive to dietary Zn intake and is therefore not suitable as biomarker for Zn status assessment. Its role as additional biomarker is uncertain. Circulating MT concentration requires further research but seems interesting as additional biomarker for plasma Zn to distinguish between

suboptimal Zn intake and redistribution of tissue Zn. Growth performance seems suitable as functional indicator to determine Zn requirements if study conditions are controlled.

Specifically for Zn status assessment in pigs, plasma Zn concentration and growth performance appears most suitable. Yet, plasma Zn concentration seems not suitable to diagnose Zn deficiency. Dietary Zn intake and serum MT concentration seems appropriate as additional biomarker for plasma Zn concentration. Liver Zn concentrations seems sensitive but contradictory observations warrant caution and bone Zn concentration is only suitable for assessing bioavailability.

Thus, a well-considered choice of the most appropriate biomarker depending on species and study objective (deficiency, requirements or bioavailability) is required for Zn status assessment in production animals. More research in controlled animal studies is warranted to 1) validate biomarkers for Zn status assessment and 2) use these biomarkers to diagnose Zn deficiency and determine Zn requirements for each species using the best available Zn sources. Possible interactions with other ingredients, especially microminerals, must be taken into account to achieve optimal animal health and minimise surplus of Zn excreted to the environment.

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Chapter 4

Fluctuations of zinc status biomarkers throughout a reproductive cycle in sows

Adapted from: Fluctuation of potential zinc status biomarkers throughout a reproductive cycle of primiparous and multiparous sows.

M.M.J. van Riet, S. Millet, E. Nalon, K.C.M. Langendries, A. Cools, B. Ampe, G. DuLaing, F.A.M. Tuyttens, D. Maes, G.P.J. Janssens

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Abstract

Fluctuations in Zn metabolism throughout gestation and lactation might affect the Zn requirements. However, scientific data on Zn requirements for breeding sows is limited. The objective of the present study was to assess the Zn status of primiparous and multiparous sows, using different Zn status biomarkers, to identify periods of critical Zn status throughout the reproductive cycle at different parities. Blood samples were taken after overnight fasting before feeding in the morning from five primiparous and ten multiparous sows at fixed time intervals during gestation (d-5, 0 (insemination), 21, 42, 63 and 84), around parturition (d108, 112, 115 (parturition) and 118), and during lactation (d122, 129 and 143 (weaning)). At parturition, blood samples were collected from two randomly selected piglets per sow before colostrum intake. Plasma was analysed for Zn and Cu content, whereas serum was analysed for alkaline phosphatase (ALP), metallothionein (MT), and albumin concentration. Independently of parity, all biomarkers fluctuated differently during gestation and lactation ($P<0.050$). This reflects their different roles in Zn metabolism, and suggests that the choice of a Zn status biomarker necessitates careful consideration. Low average plasma Zn concentrations at the end of gestation and throughout lactation seem to be replenished towards weaning.

Introduction

Fluctuations in Zn metabolism throughout the reproductive cycle of sows are a result of physiological processes during gestation and lactation to support foetal growth and development and milk synthesis (Mahan, 1990; Donangelo *et al.*, 2005; Maia *et al.*, 2007; Caulfield *et al.*, 2008). In particular, placental Zn transport, luminal Zn absorption and Zn mobilisation from the bone and liver are increased at the end of gestation and lactation (Donangelo *et al.*, 2005; Maia *et al.*, 2007; Caulfield *et al.*, 2008). This results in varying Zn transport and a changed role of transport proteins in both foetus and dam (Perveen *et al.*, 2002), thereby increasing the dietary Zn requirement.

Insufficient intake of dietary Zn during gestation and lactation may cause reproductive failures, without clinical signs of maternal Zn deficiency (Smith and Akinbamijo, 2000; Ashworth and Antipastis, 2001; Vazquez-Armijo *et al.*, 2011): the sow depletes her body Zn reserves in tissues and fluids before litter size, foetal development or milk composition are affected (Mahan, 1990; Smith and Akinbamijo, 2000; Ashworth and Antipastis, 2001; Vazquez-Armijo *et al.*, 2011). Scientific data of Zn requirements for sows is limited. Therefore, adequately measuring Zn status is critical, albeit difficult (Apgar and Fritzgerald, 1987; Wood, 2000; Hambidge, 2003).

Zinc status may be reflected by biochemical markers (*i.e.* biomarkers), which are related to the structural, regulatory and catalytic role of Zn. Plasma Zn is the most frequently used biomarker for Zn status. However, plasma Zn alone shows limitations if study conditions are not controlled or if confounding factors are present (Lowe *et al.*, 2004). These confounding factors may be infection or diseases, an acute-phase response, stress, feeding state (fasted or post prandial measures), reproductive phase (gestation), and also the method of blood collection, and concomitant blood sample transport, storage and analysis (Hambidge, 2003; Fairweather-Tait *et al.*, 2008; Lowe *et al.*, 2009). Other biomarkers such as albumin, metallothionein (MT), and alkaline phosphatase (ALP) play a role in Zn metabolism and homeostasis: Zn is bound to albumin after absorption (McDowell, 2003; Peters, 2006); MT (a small cysteine-rich protein) is involved in regulating the quantity of Zn absorbed (Cousins, 1996; McDowell, 2003; Bikker and Jongbloed, 2014); and ALP is a Zn dependent enzyme in which Zn ions are present in the active centre of the enzyme (Coleman, 1992). Together, they may be useful to assess the body's response to Zn supplementation or depletion. To our knowledge, the normal fluctuation of these biomarkers throughout the reproductive cycle of sows has not yet been determined.

The objective of the present study was to assess the Zn status of primiparous and multiparous sows using different Zn status biomarkers, to identify periods of critical Zn status throughout the reproductive cycle at different parities.

Materials and methods

Animals and diets

The experiment was performed on one group of twenty-three commercial hybrid sows (RA-SE Genetics) from the Institute for Agricultural and Fisheries Research (ILVO). However, one sow was too stressed during blood sampling and therefore excluded from the experiment. Furthermore, four sows were not gravid and three primiparous sows aborted at d58, 66 and 93 of gestation, and therefore they were not included in the analysis. One primiparous sow developed an abscess under the cheek and required veterinary treatment. This sow remained in the experimental group, because her results were not considered outliers compared with other sows. Consequently, the experiment included five primiparous and ten multiparous sows from one group that were followed during one reproductive cycle. The multiparous sows (parity ranged between 2 and 11) weighed 234± 36 (SD) kg at weaning of the preceding lactation period (*i.e.* at the start of the study, d -5). The primiparous sows (gilts, 233± 12 (SD) d old at insemination) weighed 142± 7 (SD) kg at d -5.

The sows were all housed individually during the first 4 weeks of gestation and from 1 week before parturition until weaning. During mid- and end gestation, the sows were housed as one group using an automated feeding system with individual sow recognition through an electronic transponder in the sow's ear.

The sows were fed a gestation diet and a lactation diet according to commercial dietary standards and nutrient requirements (NRC, 1998) (Table 4.1 and 4.2). Feed samples of the gestation and lactation diet were subject to proximate analysis according to international standard methods accredited by ISO 17025 (2005). The gestation diet was provided from the start of the study until 1 week before parturition, whereas the lactation diet was provided 1 week before parturition until weaning. During the first 4 weeks of gestation, the sows were fed twice daily, in total 2.3 kg. During mid- and end gestation, sows were fed 2.6 kg/d. During lactation, 0.25 kg of feed per piglet was gradually supplemented in addition to 3 kg feed, provided in two equal portions daily (*e.g.* a sow with 12 piglets received 6 kg of feed daily). There were no feed leftovers. Throughout the experiment, all sows had *ad libitum* access to drinking-water, except in the first 4 weeks of gestation, in which water was automatically provided through nipple drinkers for 15 min every hour and for 45 min while feeding in order to prohibit water spillage.

Table 4.1. Ingredient composition of the gestation and lactation diets.

Ingredients (g/kg fresh matter)	Gestation	Lactation
Beet pulp	150.0	30.0
Wheat	150.0	-
Wheat bran	120.0	49.0
Barley	150.0	107.6
Maize	125.7	350.0
Rye	-	100.0
Alfalfa meal	60.8	80.0
Soybeans heated	104.2	23.3
Soybean meal	-	156.7
Soybean oil	1.7	-
Rapeseed meal	70.0	-
Beet molasses	40.0	30.0
Premix 1%*	10.0	10.0
Benzoic acid	-	5.0
Limestone	8.5	12.2
Calcium monophosphate	3.3	13.2
Lard	-	22.3
Salt	5.0	4.3
L-Lys HCL	0.7	2.8
L-Thr	0.1	1.6
L-Val	-	1.2
DL-Met	-	0.4
L-Trp	-	0.3
Phytase†	0.1	0.1

* Premix1% included per kg diet: vitamin A (12000 IU), vitamin D3 (2000 IU), vitamin E (75.0 mg), vitamin K3 (1.01 mg), vitamin B1 (1.5 mg), vitamin B2 (5.0 mg), vitamin B5 (18.0 mg), vitamin B6 (4.0 mg), vitamin B12 (0.03 mg), vitamin B3 (25.0 mg), vitamin B11 (3.0 mg), biotin (0.3 mg), choline (432.5 mg), $FeSO_4*H_2O$ (Fe: 150.0 mg), $CuSO_4*5H_2O$ (Cu: 16.43 mg), MnO (Mn: 50.0 mg), ZnO (Zn: 92.97 mg), $Ca(IO_3)_2$ (I: 2.0 mg), Na_2O_3Se (Se: 0.40 mg), Ca (0.89 mg), P (0.05 mg), Mg (0.16 mg), Na (3.02 mg), Cl (0.12 mg), K (0.08 mg), S (0.11 mg), Lysine (0.04 mg), Methionine (0.01 mg), Threonine (0.03 mg), Tryptophan (0.01 mg), Butylhydroxytoluene (0.50 mg), Ethoxyquine (0.55 mg), Butylated hydroxy anisol (0.05 mg).
† Phytase level is 5000 FTU/g.

Table 4.2. Analysed and calculated* nutrient composition of the gestation and lactation diets.

Chemical analysis (g/kg)	Gestation	Lactation
DM	885.6	893.2
Crude ash	58.1	62.1
Crude protein	151	164.9
Crude fat	51.3	55.7
Starch	295.6	355.1
Sugar	63.1	58.6
ADF	86.3	55.9
NDF	182.7	122.6
ADL	14.3	8.3
Ca	9.5	12.1
P	4.7	6.0
Cu (mg/kg)	19.9	24.8
Zn (mg/kg)	124.2	124.5
ID Lys	5.2	7.9
ID Met	1.8	2.3
ID Thr	3.4	5.5
ID Trp	1.2	1.6
ID Arg	6.7	8.1
ID Leu	7.8	10.0
ID Ile	3.9	4.8
ID His	2.8	3.2
ID Val	4.7	6.7
ID Phe	4.7	6.0
NEv (MJ/kg)	8.6	9.2

ADF, acid-detergent fibre; NDF, neutral-detergent fibre; ADL, acid-detergent lignin; ID, ileal digestible; NEv, net energy for pigs.

* Chemical analyses of ID amino acids and NEv are calculated according to the feed tables of the Centraal Veevoederbureau (CVB, The Netherlands), 2007.

Experimental design

Blood samples were taken before feeding in the morning (between 08.00-10.00 hours), after overnight fasting (17.5 h), at fixed time intervals throughout one reproductive cycle: d-5 (weaning of the preceding cycle for multiparous sows; “start of the study in December 2011”); throughout gestation (d0 (insemination), 21, 42, 63 and 84); around parturition (d108, 112, 115 (parturition) and 118); and during lactation (d122, 129 and 143 (weaning)). The days until parturition were predicted from the expected date of parturition (based on a gestation period of 115d), whereas from parturition onwards, the sows were sampled based on the date of parturition. Blood samples were

collected from the jugular vein using stainless-steel needles and plastic syringes, and added to one heparin and one serum vacuum tube (Terumo Europe, Leuven, Belgium). At parturition, two piglets per sow were randomly selected for blood sampling from the jugular vein before colostrum intake, and the blood sample was added to one heparin vacuum tube (Terumo Europe, Leuven, Belgium).
Within 1 h after the first piglet was born, colostrum was collected. At 1 week after parturition, milk samples were also collected, using 1 mL oxytocin (10 IU oxytocin/mL (*i.e.* 17µg/mL); VMD N.V., Arendonk, Belgium) to stimulate milk release. The colostrum and milk samples were collected from all nipples from either the left or right side of the udder, repeatedly from front to back nipples, until 20 mL was collected. At weaning, reproductive performances were documented, taken into account cross-fostering and provision of creep feed (transitional feed) to the piglets from d10 postpartum.
Bodyweight and backfat thickness were measured at the start of the study (*i.e.* weaning of the preceding lactation period for the multiparous sows (d-5)), at d63, 84 and 108 of gestation, and at weaning (d143). Backfat thickness was determined between the 3rd and 4th last rib, 7 cm at the left and right side of the vertebrae (P2). After P2 was lubricated, backfat measures were repeated 3 times, alternately at the left and right side (PIGLOG 105, SFK-Technology, Søborg, Denmark). The average thickness was used for further calculations.
The present observational study was conducted according to the institutional and national guidelines for the care and use of animals and all experimental procedures involving these animals were approved by ILVO's ethical committee for animal experiments (approval no. 2010/160, August 16th, 2011).

Preparation of blood samples

A heparinised blood sample (1 mL) was used for haematocrit assay and centrifuged at 2749 *g* for 30 min at 20 ºC. The remainder was centrifuged at 1500 *g* for 10 min at 4 ºC, divided over different 5 mL disposable polystyrene tubes, and stored for 24 h at -20 ºC, thereafter at -80 ºC until analysis. Plasma was analysed for Zn and Cu concentrations.
After overnight incubation at 4 ºC, the serum was separated by centrifugation at 1500 *g* for 10 min at 4 ºC and stored for 24 h at -20 ºC, thereafter at -80 ºC until analysis. Serum was analysed for ALP, albumin and MT concentrations. Colostrum, milk and the piglets' plasma were analysed for Zn and Cu concentrations. Plasma Cu concentration was not measured as a Zn status biomarker; however, because it uses some of the same transporter mechanisms, it may provide information on the affected pathways (Milne *et al.*, 2001; McDowell, 2003; Bikker and Jongbloed, 2014; EFSA, 2014).

Analysis of zinc status biomarkers

Plasma samples were deproteinated (Randox ZN2607, Randox Laboratories Ltd., Crumlin, UK) by mixing with an equal volume of trichloroacetic acid (TCA) and centrifuged at 10,000 *g* for 10 min. The remaining supernatant was used within 2 h to determine plasma Zn and Cu concentrations.

For *plasma Z*n analysis, the deproteinated plasma was diluted 5 times with a colour reagent (Randox kit, ZN2341, Randox Laboratories Ltd., Crumlin, UK), and incubated for 5 min at 25 ºC. Absorbance was measured at a wavelength of 570 nm with a reference wavelength of 620 nm using a microplate reader (EZ reader 400, Biochrom Ltd., Cambridge, UK). Plasma Zn concentration was interpolated from the standard multipoint calibration curve. The inter- and intra-assay CV were 2.19 and 3.61 %, respectively. For quality control, pooled porcine plasma samples were spiked using $ZnCl_2$ to calculate the rate of Zn recovery. The regression line of the calibration curve to compare the measured and expected Zn concentration was as follows: $y = 0.0003x + 0.0252$, $R^2 = 0.9966$. The minimum and maximum recovery rates were 99.5 and 119.8 %, respectively.

For *plasma Cu* analysis, a reagent (Randox kit, CU2340, Randox Laboratories Ltd., Crumlin, UK) was added to the deproteinated plasma, and this solution was incubated for 60 s at 37 ºC (Memmert Elanco incubation oven, Memmert GmbH, Schwabach, Germany). Absorbance was measured at a wavelength of 580 nm (Ultrospec IIE, LKB Biochrom Ltd., Cambridge, UK). A colour reagent was added and the solution was mixed. After 5 min of incubation at 37 ºC, absorbance was measured at the same wavelength. Plasma Cu concentration was interpolated from the standard multipoint calibration curve. The inter-assay CV was 2.83 %. For quality control, pooled porcine plasma samples were spiked using $CuCl_2*2(H_2O)$ to calculate the rate of Cu recovery. The regression line of the calibration curve to compare the measured and expected Cu concentration was as follows:
$y = 0.0006x + 0.0806$, $R^2 = 0.9912$. The minimum and maximum recovery rates were 96.4 and 103.7 %, respectively.

Albumin was determined colourimetrically (Cobas integra Albumin Gen.2, Roche Diagnostics GmbH, Mannheim, Germany), by binding to an anionic dye (bromcresol green) to form a blue-green complex. The total albumin concentration was proportional to the colour intensity and determined by an increase in absorbance at 583 nm. Serum albumin concentrations were within the expected range of the kit (3- 96 g/L), and the recovery rate was within 10 % of the initial value at an albumin concentration of 35 g/L. The observed albumin concentrations were daily controlled using an independent human control serum with a high and low known concentration.

Metallothionein was determined using competitive ELISA (Porcine Metallothionein (MET) Elisa kit, E07M0030, BlueGene Biotech CO., Shanghai, China), including duplicate standards and controls from human origin for each run. The product of the enzyme-substrate reaction forms a

blue-coloured complex and turns into yellow after the reaction was stopped by adding a stop solution. The intensity of the colour was measured spectrophotometrically at 450 nm (BEP 2000, Siemens AG, Munich, Germany). Metallothionein concentration was interpolated from the standard curve. The certificate of analysis reported an inter- and intra-assay CV of 6.6- 8.2 % and 4.4- 5.6 %, respectively. The recovery rates ranged between 94 and 103 %.

Alkaline phosphatase was determined colourimetrically (Cobas c systems ALP2, Roche Diagnostics GmbH, Mannheim, Germany). In the presence of Mg and Zn, *p*-nitrophenyl phosphate was cleaved into phosphate and *p*-nitrophenol. The released *p*-nitrophenol was directly proportional to the catalytic ALP activity, which is determined by an increase in absorbance at 480/450 nm. The device was calibrated with a known concentration before the analysis to measure the concentration of ALP. Serum ALP concentrations were within the expected range of the kit (5- 1200 U/L), and the recovery was within 10 % of the initial value at an ALP concentration of 100 U/L. The observed ALP concentrations were daily controlled using an independent human control serum with a high and low known concentration.

Concentration of zinc and copper in colostrum and milk

Colostrum and milk samples (1.5 g) were digested in a microwave oven (MarsX, CEM, Matthews, NC, USA), using HNO_3/H_2O_2, and analysed for Zn and Cu concentrations by inductively coupled plasma optical emission spectrometry (ICP-OES, Vista MPX, Varian Inc., Palo Alto, CA, USA). For quality control, two cows' milk samples were spiked, both with 50 µg/L and with 100 µg/L of Cu and Zn. The recovery rates of spiked Cu and Zn varied between 81.2 and 88.9 %, and between 83.7 and 91.2 %, respectively.

Statistical analysis

The effect of parity on bodyweight, backfat thickness and reproductive performances was analysed using a linear model with performance as the outcome and parity as fixed effect. The evolution over time of bodyweight and backfat thickness were analysed using a linear mixed model with bodyweight or backfat thickness as the outcome and parity as fixed effect. Sow was added as random effect to correct for repeated measures within sows.

The evolution over time during gestation (period between d0 (insemination) and d115 (parturition)) and lactation (period between d118 and 143 (weaning)) of all biomarkers were analysed using a linear mixed model for each reproductive phase (gestation and lactation), with the biomarker as the outcome and parity, haematocrit, time, interaction between parity and time, and a quadratic time effect as fixed effects. Inclusion of a quadratic time effect allows the identification of a non-linear

evolution over time. In case of repeated measures, a random effect for sow was introduced in the model.

Biomarkers in colostrum, milk and the piglets' plasma were analysed using a linear mixed model, with the biomarker as the outcome and all sows' biomarkers at parturition or the average during gestation, and parity as fixed effects. The non-significant biomarkers were removed from the model. Sow was introduced as a random effect to correct for clustering of piglets within sows.

The analysed data were considered sufficiently normally distributed based on the graphical evaluation (histogram and QQ-plot) of the residuals. In case of *post hoc* pairwise testing, p-values were corrected with the Tukey-Kramer adjustment for multiple comparisons. All analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

The bodyweight of multiparous sows increased during gestation and decreased after parturition to similar values at weaning of the preceding cycle (Table 4.3). Primiparous sows showed a lower bodyweight compared with multiparous sows throughout the reproductive cycle, and had a higher bodyweight at weaning compared with the start of the study (Table 4.3). Backfat thickness decreased during lactation ($P<0.001$), independent of parity. No significant parity effect was found in reproductive performances (Table 4.4).

Table 4.3. Bodyweight and backfat thickness throughout the reproductive cycle of sows (n= 15; 5 primiparous and 10 multiparous sows).

Day of reproductive cycle*	Bodyweight (kg)				Backfat (mm)			
	Primiparous		Multiparous		Primiparous		Multiparous	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
-5	$142^{a,A}$	3	$234^{a,B}$	11	18^{a}	2	18^{ab}	1
63	$188^{b,A}$	4	$250^{b,B}$	11	21^{ab}	2	19^{a}	2
84	$203^{c,A}$	6	$269^{c,B}$	11	21^{ab}	1	19^{a}	1
108	$223^{d,A}$	6	$279^{d,B}$	11	23^{b}	1	18^{a}	1
143	$169^{e,A}$	6	$237^{a,B}$	10	14^{c}	1	15^{b}	2
P								
Day	<0.001				<0.001			
Parity	<0.001				0.373			
D×P†	<0.001				0.117			

* Day -5 represent the start of the study, d63, 84 and 108 represent gestation and d143 represents weaning.

† Interaction between day (D) and parity (P).

[a-e] Mean values within a column with unlike superscript letters were significantly different between different days of the reproductive cycle ($P<0.050$).

[A,B] Mean values within a row with unlike superscript letters were significantly different between the primiparous and multiparous sows for the specific day of the reproductive cycle ($P<0.050$).

Table 4.4. Reproductive performance of primiparous (n= 5) and multiparous (n= 10) sows*.

Performance characteristics	Primiparous		Multiparous		
	Mean	SEM	Mean	SEM	*P*
Piglets born alive (n)	13.0	1.2	13.0	0.9	0.741
Stillborn piglets (n)	1.0	0.4	1.0	0.4	0.537
Average bodyweight piglets born alive (kg)	1.3	0.1	1.5	0.1	0.210
Piglet mortality (%)	13.0	5.8	10.0	2.4	0.537
Weaned piglets (n)	11.0	0.9	12.0	0.9	0.480
Average bodyweight weaned piglets (kg)	8.9	0.2	8.4	0.2	0.153

* No significant differences were found between the primiparous and multiparous sows ($P>0.050$).

The haematocrit level (Figure 4.1) did not fluctuate significantly throughout gestation (β= 0.019, P=0.955) and lactation (β= -0.107, P=0.581), despite the numerically increased percentage at d21 of gestation. There was also no parity effect for haematocrit during gestation (P=0.458) and lactation (P=0.955). The concentrations of plasma Zn (P=0.173 for gestation and P=0.389 for lactation), serum MT (P=0.127 for gestation and P=0.514 for lactation), plasma Cu (P=0.314 for gestation and P=0.259 for lactation) and serum ALP (P=0.346 for gestation and P=0.738 for lactation) were not associated with the haematocrit level. Fluctuation in albumin levels tended to be positively associated with the haematocrit level during gestation (P=0.086) and lactation (P=0.049).

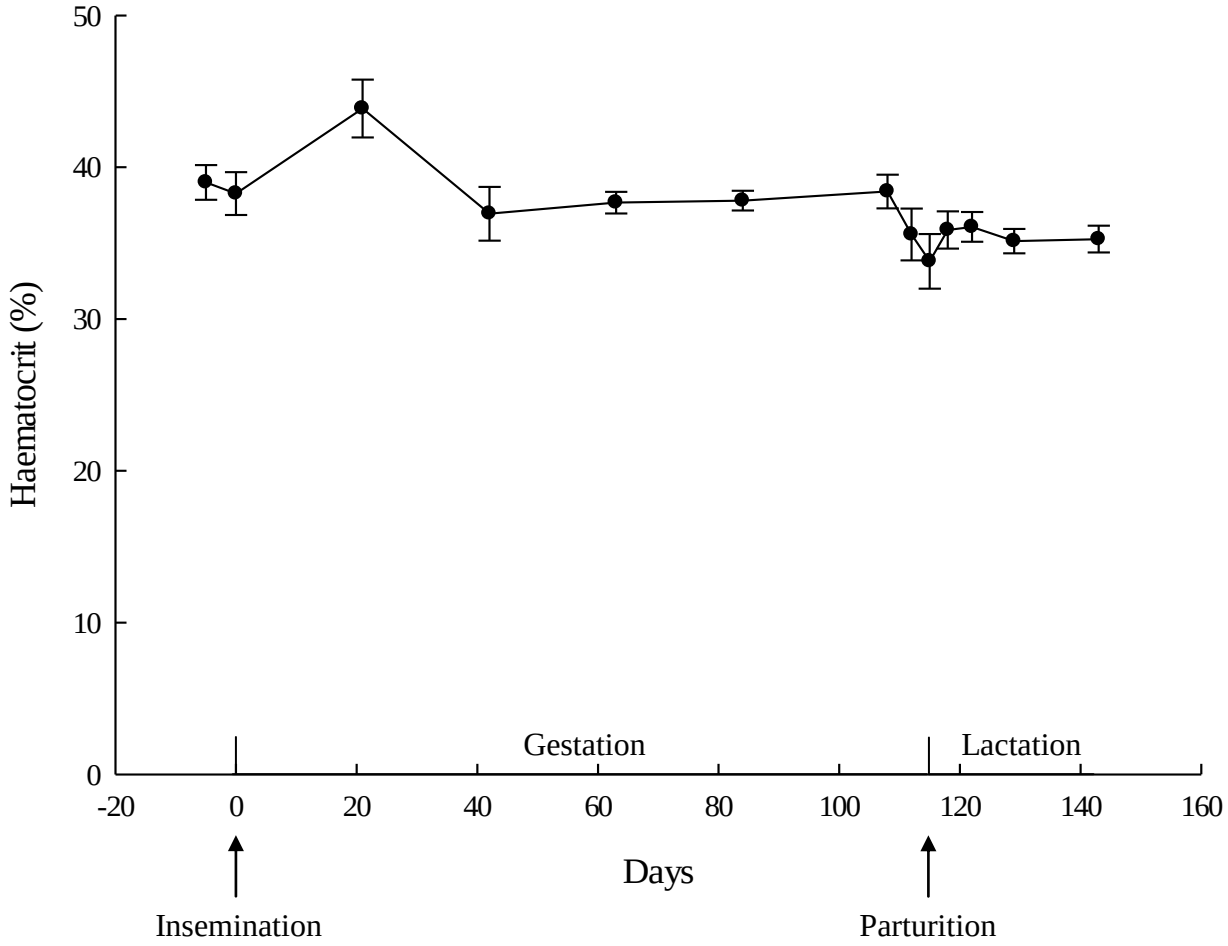

Figure 4.1. Haematocrit levels (%) throughout gestation and lactation in sows (n 15; 5 primiparous and 10 multiparous sows). Values are means, with their standard errors represented by vertical bars. During gestation and lactation, no fluctuation was observed (P=0.955 and P=0.581, respectively).

Despite the parity effect for bodyweight and a tendency for an interaction between parity and serum albumin concentration during gestation, the fluctuations of the other biomarkers did not differ between primiparous and multiparous sows. Therefore, average biomarker fluctuations are presented in the figures.

Plasma Zn concentration decreased linearly after insemination (β= -1.934, P<0.001) and increased quadratically towards parturition (β= 0.012, P<0.001) (Figure 4.2). After parturition, plasma Zn concentration seemed to first increase, then decrease and increase again during the last 14d of the lactation period. However, there was only a tendency for a quadratic time effect for the last 14d of lactation (β= 0.118, P=0.063). The variation between sows was high, especially at d-5, 42 and 112 of gestation, and at weaning (d143).

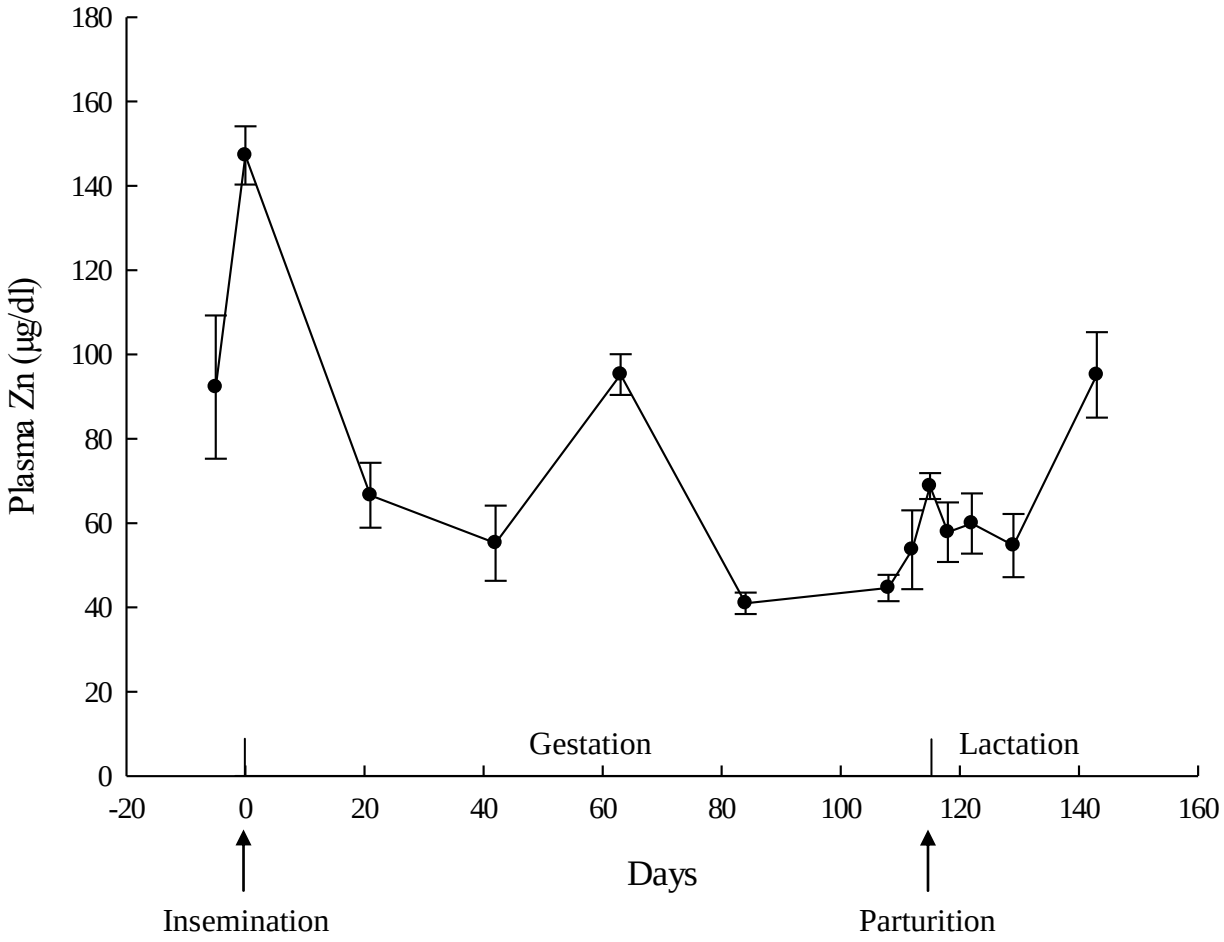

Figure 4.2. Plasma zinc concentrations (µg/dL) throughout gestation and lactation in sows (n 15; 5 primiparous and 10 multiparous sows). Values are means, with their standard errors represented by vertical bars. During gestation, plasma Zn concentration decreased linearly after insemination (P<0.001) and increased quadratically towards parturition (P<0.001). During lactation, plasma Zn concentration tended to increase quadratically at the end of lactation (P=0.063). To convert values from µg/dL to µmol/L, multiply by 0.153.

A distinct fluctuation in plasma Cu concentration during gestation was not found (β= 0.408, P=0.127, Figure 4.3). Plasma Cu concentration decreased linearly during lactation (β= -6.716, P<0.001) and increased quadratically towards weaning (β= 0.126, P=0.024).

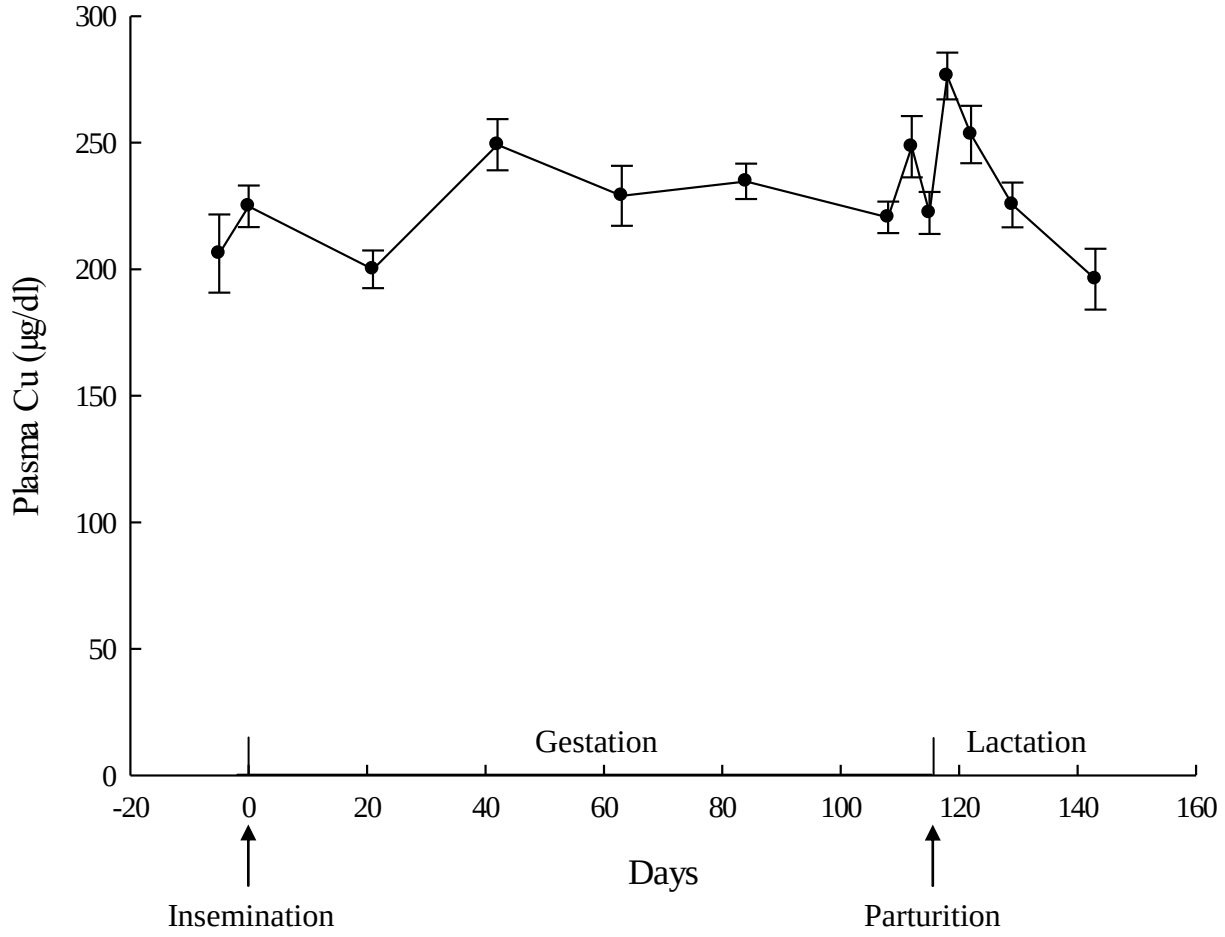

Figure 4.3. Plasma copper concentrations (µg/dL) throughout gestation and lactation in sows (n 15; 5 primiparous and 10 multiparous sows). Values are means, with their standard errors represented by vertical bars. During gestation, no fluctuation was observed for plasma Cu concentration (P=0.127). During lactation, plasma Cu concentration decreased linearly (P<0.001) and increased quadratically towards weaning (P=0.024). To convert values from µg/dL to µmol/L, multiply by 0.157.

The total serum albumin concentration tended to decrease linearly during the beginning of gestation, though it tended to decrease less for primiparous sows (tendency for an interaction between parity and day of gestation, P=0.055). A quadratic time effect was found towards the end of gestation (β= 0.000, P<0.001, Figure 4.4). During lactation, serum albumin concentration decreased linearly (β= -0.039, P=0.018).

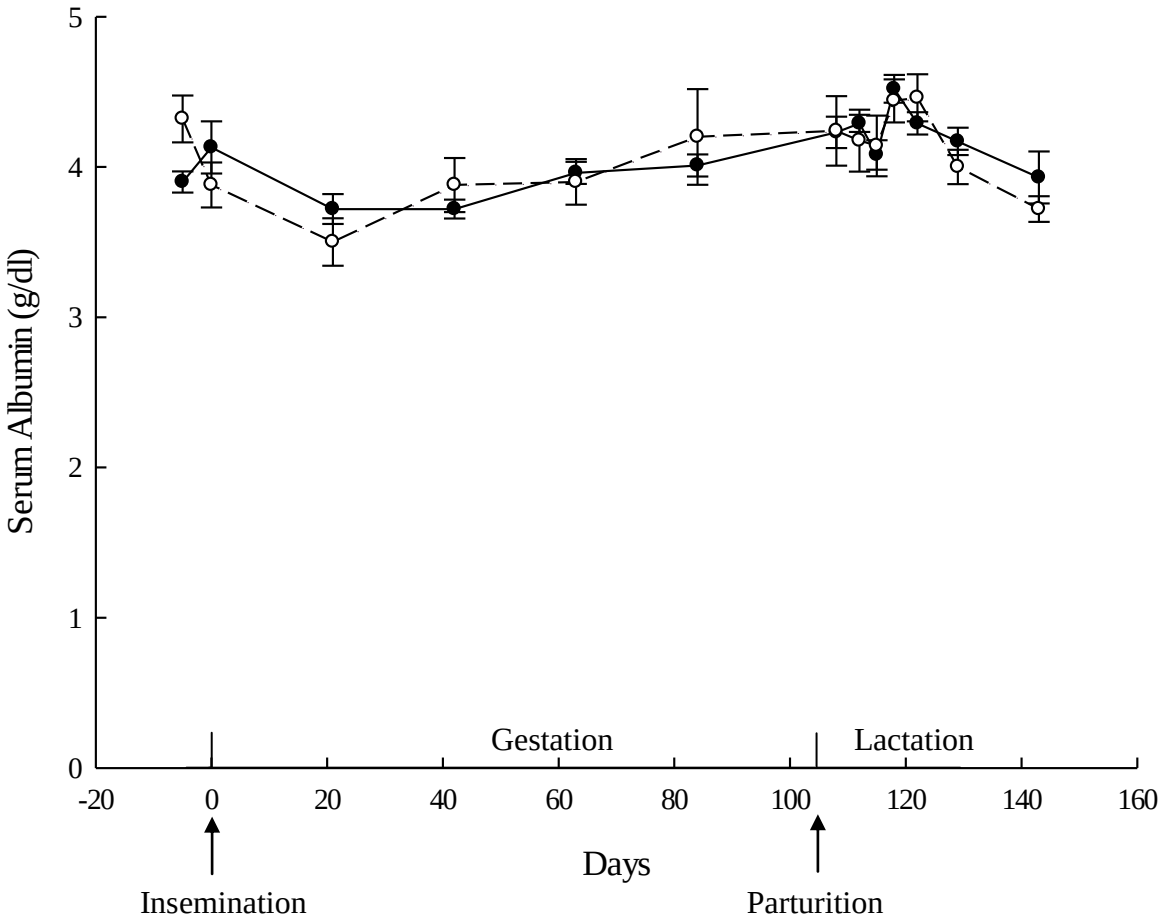

Figure 4.4. Total serum albumin concentrations (g/dL) throughout gestation and lactation in sows (n 15; 5 primiparous (–○–) and 10 multiparous (–●–) sows). Values are means, with their standard errors represented by vertical bars. During gestation, serum albumin concentration tended to decrease linearly at the beginning of gestation, though tended to decrease less for primiparous sows (tendency for an interaction between parity and day of gestation, P=0.055) and increased quadratically towards the end of gestation (P<0.001). During lactation, serum albumin concentration decreased linearly (P=0.018). To convert values from g/dL to µmol/L, multiply by 10.

During gestation, the serum MT concentration did not change significantly (β= -0.016, P=0.162) (Figure 4.5). Shortly after parturition, MT concentration decreased linearly (β= -0.244, P=0.001) and increased quadratically towards the end of lactation (β= 0.007, P<0.001).

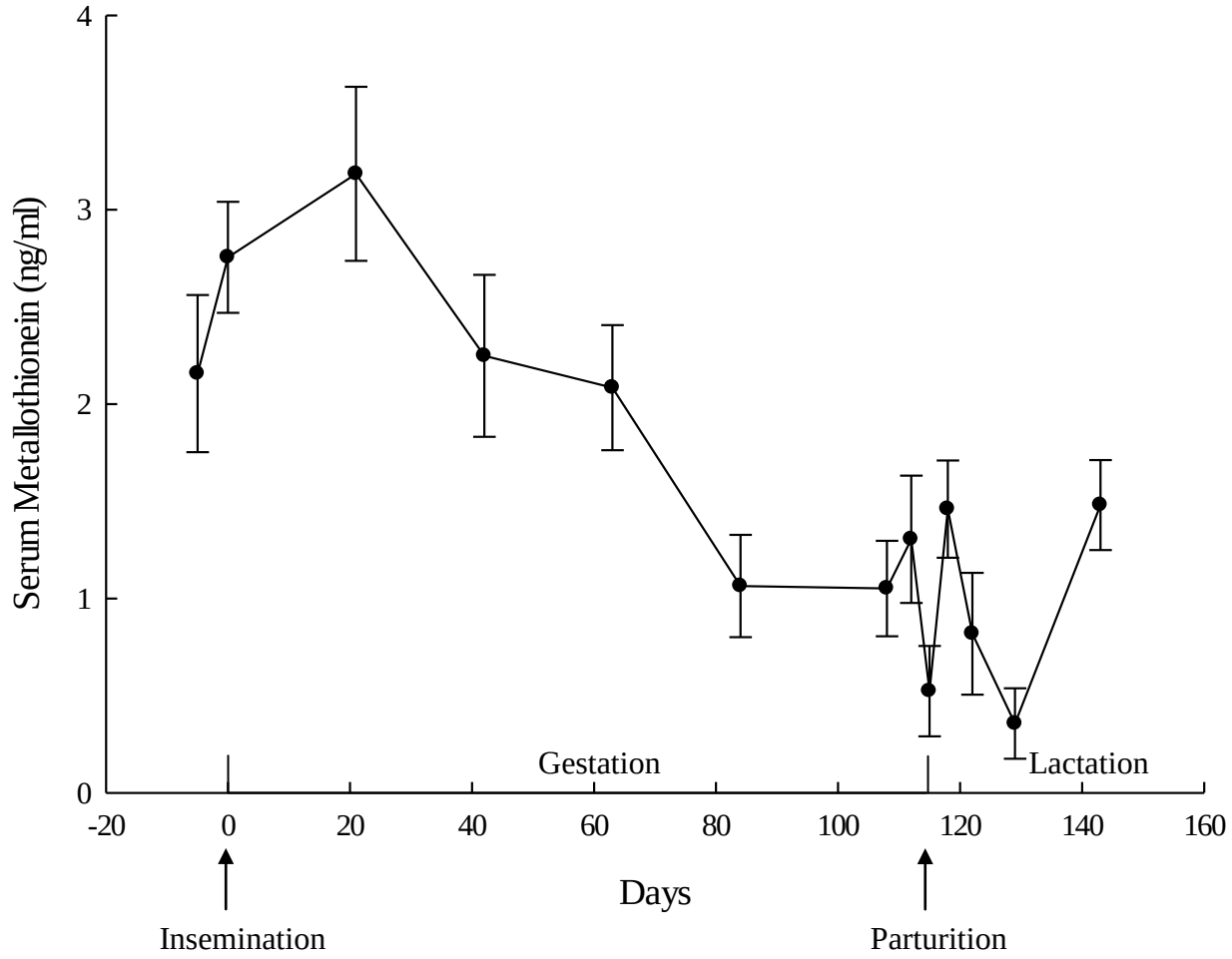

Figure 4.5. Serum metallothionein (MT) concentrations (ng/mL) throughout gestation and lactation in sows (n 15; 5 primiparous and 10 multiparous sows). Values are means, with their standard errors represented by vertical bars. During gestation, no fluctuation was observed for serum MT concentration (P=0.162). During lactation, serum MT concentration decreased linearly (P=0.001) and increased quadratically towards the end of lactation (P<0.001).

Serum ALP concentration increased linearly at the beginning of gestation (β= 0.395, P=0.002) and decreased quadratically towards parturition (β= -0.003, P=0.001) (Figure 4.6). During the lactation period, no significant fluctuations were observed in ALP concentration (β= 0.859, P=0.140). No parity effect was found, despite the numerically higher serum ALP concentrations of primiparous sows throughout the reproductive cycle (P=0.102 for gestation and P=0.473 for lactation).

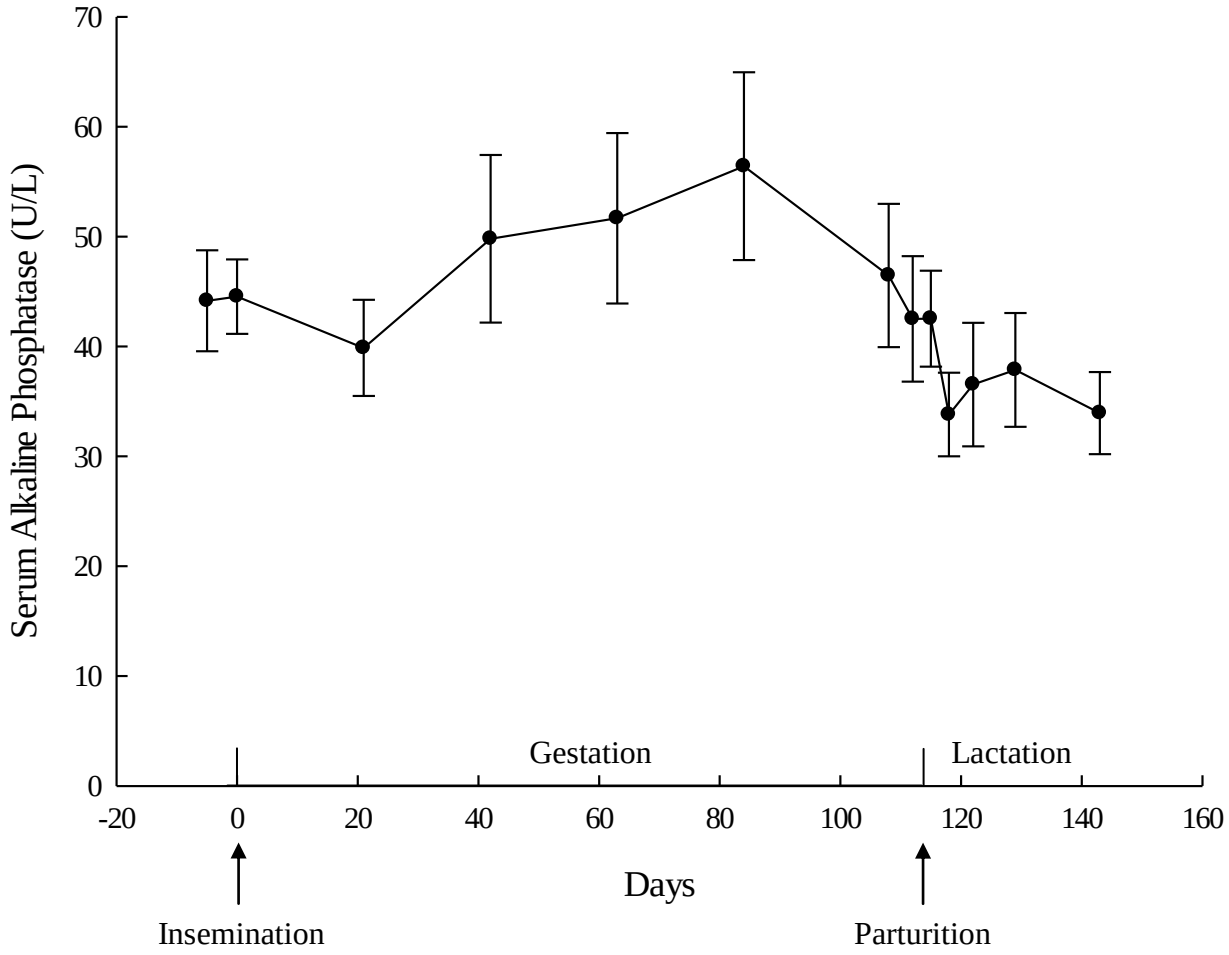

Figure 4.6. Serum alkaline phosphatase (ALP) concentrations (U/L) throughout gestation and lactation in sows (n 15; 5 primiparous and 10 multiparous sows). Values are means, with their standard errors represented by vertical bars. During gestation, serum ALP concentration first increased linearly (P=0.002) and decreased quadratically towards parturition (P=0.001). During lactation, no fluctuation was observed for serum ALP concentration (P=0.140).

Biomarkers in piglets' plasma, colostrum and milk

No significant parity effects were found for piglet bodyweight, haematocrit level, plasma Zn and Cu concentrations, placental weight, colostrum Zn and Cu concentrations, and milk Zn concentration (P>0.050) (Table 4.5). Milk Cu concentration tended to be lower for primiparous than for multiparous sows (β= -0.378, P=0.099).

At parturition, serum albumin concentration of the sow tended to be associated with the average bodyweight of her piglets born alive (β= 0.297, P=0.060).

Plasma Zn concentration of the piglets was associated with serum MT concentration of the sow during gestation and at parturition (β= 11.891, P=0.036 and β= 14.261, P=0.028, respectively) and associated with the haematocrit level of piglets (β= -1.732, P=0.032).

Table 4.5. Piglets' indicatives (n= 30) and colostrum and milk zinc and copper concentrations.

Indicatives*	Primiparous		Multiparous		*P*
	Mean	SE	Mean	SE	
BW (kg)	1.5	0.1	1.6	0.1	0.172
HCt level (%)	37.9	2.0	41.6	1.2	0.676
Plasma Zn					0.268
µg/dL	73.0	8.9	60.7	5.9	
µmol/L	11.2	1.4	9.3	0.9	
Plasma Cu					0.888
µg/dL	12.6	1.2	15.6	2.1	
µmol/L	2.0	0.2	2.4	0.3	
Placenta weight (kg)	2.6	0.5	2.7	0.2	0.951
Colostrum (mg/kg)					
Zn	18.4	2.0	16.2	1.2	0.529
Cu	4.5	0.2	4.4	0.3	0.564
Milk (mg/kg)					
Zn	6.7	0.2	6.6	0.2	0.948
Cu	1.1	0.1	1.4	0.1	0.099

BW, bodyweight; HCt, haematocrit level.
* Piglets' BW, HCt, and plasma Zn and Cu concentrations were from two randomly selected piglets per sow at parturition (d115) before colostrum intake.

Discussion

Fluctuations of Zn status biomarkers during gestation and lactation differed distinctly between biomarkers and were independent of parity (except for serum albumin). These differences are probably related to the respective roles of these biomarkers in Zn homeostasis throughout the different phases of the reproductive cycle, as has been shown in other animal species and human subjects. However, the observed evolution over time might have been partly influenced by random period effects, such as temperature.

During gestation, Zn requirements are increased to support embryogenesis and foetal growth and development (Mahan, 1990; King, 2000; Donangelo *et al.,* 2005; Maia *et al.,* 2007; Caulfield *et al.,* 2008; Donangelo and King, 2012), resulting in homeostatic adjustments to (re)distribute more Zn to maternal tissues (*e.g.* placenta, liver and bone) and to the foetus (King, 1990; Tamura and Goldenberg, 1996; Krebs, 1998; Donangelo and King, 2012). This Zn sequestration to maternal tissues and foeti may decrease the total circulating Zn concentration (Donangelo and King, 2012).

In the present study, plasma Zn concentration decreased during gestation as hypothesised based on other studies in sows (Hill *et al.,* 1983a; Kalinowski and Chavez, 1984; Girard *et al.,* 1996). This conceivably reflects the increased intracellular Zn need to protect maternal erythrocytes from oxidative stress, thereby increasing the erythrocyte Zn and MT concentrations (due to the synthesis

of carbonic anhydrase) and decreasing plasma Zn concentrations (Tamura and Goldenberg, 1996; Caulfield *et al.*, 1999; Donangelo and King, 2012).

Zinc is predominantly bound to albumin after absorption and has a high affinity to albumin (McDowell, 2003; Peters, 2006). A lower Zn fraction bound to albumin or a lower affinity of Zn for albumin was mentioned to explain the decreased plasma Zn concentration. Interestingly, the total serum albumin concentration in the present study increased during gestation, hence not reflecting the decline in Zn concentration, as expected and as has been found in other studies in women (Hambidge *et al.*, 1983; Maia *et al.*, 2007).

Whereas albumin serves as an important Zn transporter, MT is involved in Zn transfer within the intestinal mucosal cell, regulating the quantity of Zn absorbed (Cousins, 1996; McDowell, 2003; Bikker and Jongbloed, 2014). When Zn is bound to albumin in plasma and entering the liver, MT is synthesised. Metallothionein in the liver removes Zn from the plasma and redistributes it to various tissues (Bremner, 1993; King, 2000; McDowell, 2003). Hepatic MT synthesis is influenced by dietary Zn intake as well as plasma Zn concentrations (Cousins, 1996; Richards, 1999; McDowell, 2003; Gibson, 2005b), suggesting that serum MT concentration reflects Zn absorption. This is confirmed in the present study, where serum MT fluctuated similarly to plasma Zn concentrations during gestation but not at parturition when the dietary Zn intake is low.

Plasma Cu can be bound to MT as well, but fluctuations in plasma Cu concentration in the present study are comparable with other studies in sows (Girard *et al.*, 1996; Richards, 1999), women (Álvarez *et al.*, 2007) and ewes (Elnageeb and Adelatif, 2010), and therefore suggests that plasma Cu was not interfering with the fluctuation of serum MT. This was also observed by López-Alonso *et al.* (2012a,b), where liver and kidney MT concentrations were highly dependent on the Zn status of the pig, and neither Cu nor Cd displaced Zn from MT.

Alkaline phosphatase is found in the blood and bone and is involved in the formation of hydroxyapatite (Peters, 2006; van Riet *et al.*, 2013), which is important for regulating ordered mineral deposition during bone formation (Ruz *et al.*, 1992; Clarke, 2008; van Riet *et al.*, 2013). Alkaline phosphatase concentration increased during gestation and decreased towards parturition in the present study. This fluctuation may indicate that ALP is related to bone formation in the process of bone remodelling.

During lactation, Zn requirements are increased to support milk synthesis and secretion (Donangelo and King, 2012), resulting in a (re)distribution of Zn to the mammary gland. This occurs by mobilising Zn from involuting tissues (uterus) and maternal blood after parturition, mobilisation from the bone, and by increased efficiency of Zn absorption in the intestine (Swanson and King,

1983; Moser-Veillon, 1995; Tamura and Goldenberg, 1996; Krebs, 1998; Donangelo and King, 2012). The distribution of Zn to the mammary gland may affect Zn-related blood parameters differently than during gestation (Donangelo and King, 2012).

Towards the end of lactation, plasma Zn concentrations in the present study seemed to return to initial values at early gestation, probably resulting from increased absorption or decreased nutrient requirements (Liesegang *et al.,* 2006; Donangelo and King, 2012). These plasma Zn fluctuations during lactation correspond to other studies in sows (Girard *et al.,* 1996), women (Donangelo *et al.,* 2005) and sheep (Gürdoğan *et al.,* 2006), except for the study by Kalinowski and Chavez (1984), who did not observe an increase in plasma Zn concentration during lactation in sows. However, in that study, no blood samples were analysed after 2 weeks of lactation.

Similarly, to fluctuation in serum albumin concentrations during gestation, total serum albumin concentrations did not reflect the changes in plasma Zn concentration during lactation in the present study: the increase in plasma Zn concentrations coincided with an increase in serum albumin concentrations. Zinc is then rapidly removed from albumin and transferred to other tissues, thereby ensuring the capacity to maintain Zn homeostasis. However, this was not demonstrated in the present study or other studies in women (Hambidge *et al.,* 1983; Maia *et al.,* 2007).

Serum MT concentrations increased at the end of lactation in the present study. This increase agrees with increased Zn absorption, in which more plasma Zn enters the liver where hepatic MT is synthesised to sequester Zn from the plasma to the tissues.

The fact that plasma Cu concentrations decreased towards the end of lactation, as opposed to plasma Zn concentrations seems logical as Zn is an important antagonist of Cu. This corresponds also to other studies in sows (Kalinowski and Chavez, 1984; Girard *et al.,* 1996) and ewes (Elnageeb and Adelatif, 2010).

The mobilised and absorbed Zn will be used for milk production to provide Zn to the piglets. Plasma Zn concentration in piglets before colostrum intake may reflect the dietary Zn intake levels of sows during gestation (Henkin *et al.,* 1971; Dreosti *et al.,* 1982; Hill *et al.,* 1983c; Mahan, 1990; Peters, 2006). However, in the present study, maternal plasma Zn concentration was not associated with the piglets' plasma Zn concentration. Interestingly, maternal serum MT concentration was significantly associated with the piglets' plasma Zn concentration: higher MT concentrations in sows during gestation and at parturition increased plasma Zn concentration in the piglets' plasma.

Additionally, fluctuations of Zn status biomarkers may identify critical periods throughout the reproductive cycle during which high Zn requirements are not met by dietary Zn intake. It has been shown that Zn deficiency during gestation may affect the growth and development of the foetus,

induce complications at birth, and limit the metabolic adaptation capacity of the sow (Cherry *et al.*, 1981; Apgar and Fitzgerald, 1985; Swanson and King, 1987; Álvarez *et al.*, 2007). In literature, marginal values for plasma/serum Zn concentrations for pigs have been suggested (Suttle, 2010), with a lower limit of normality of 60 µg/dL (9.2 µmol/L) and a normal marginal range for mean serum concentrations between 40 and 70 µg/dL (6.1-10.7 µmol/L). In the present study, the average plasma Zn concentration was 71.5 µg/dL (10.9 µmol/L) during gestation and 66.9 µg/dL (10.2 µmol/L) during lactation. The sows in the present study had on average sufficient plasma Zn levels. However, at d42, 84, 108, 112, 118, 122 and 129, the average plasma Zn concentrations were below the normal range defined by Suttle (2010), though sows can replace their Zn stores towards weaning. The dietary Zn concentration was above the Zn requirements, and no conclusion can be drawn for the presence of marginal Zn deficiency. Furthermore, plasma Zn concentrations varied widely among sows, which also hamper sound conclusions in individual sows.

Conclusion

Zinc status shows distinct fluctuations throughout the reproductive cycle of sows, independently of parity. However, this Zn status pattern differs according to the chosen Zn status biomarker. Therefore, assessing Zn status in breeding sows should best be determined with multiple biomarkers. For the present study, a combination of plasma Zn and serum MT seemed most suitable to assess the Zn status.

Sound conclusions regarding critical periods for Zn status throughout the reproductive cycle in sows could not be distinguished in the present study. For future studies, it is good to take into account that fluctuations in plasma Zn concentrations over a reproductive cycle are noticeable. After periods of lower plasma Zn concentration at the end of gestation and throughout lactation, plasma Zn concentrations increase towards weaning.

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Chapter 5

Protein and zinc source

Adapted from: No indications that zinc and protein source affect Zn bioavailability in sows during late gestation fed adequate dietary Zn concentrations.

M.M.J. van Riet, S. Millet, E-J Bos, E. Nalon, B. Ampe, L. Sobry, F.A.M. Tuyttens, D. Maes, G. Du Laing, T. Nagels, G.P.J. Janssens.

Submitted

Abstract

Previous *in vitro* research has shown the possibility of spontaneous chelation of Zn in the presence of easily digestible protein sources. The objective of this study was to investigate the possible interaction between zinc (Zn) source and protein source on the *in vivo* Zn bioavailability in sows during late gestation that were fed adequate dietary Zn concentrations. Fifty-six sows were randomly allocated to one of four dietary treatment groups during a 20-day experimental period: 1) organic Zn + soybean meal, 2) inorganic Zn + soybean meal, 3) organic Zn + hydrolysed feather meal, and 4) inorganic Zn + hydrolysed feather meal. Zinc was provided at adequate dietary Zn concentrations, in which organic Zn was added as a Zn amino acid complex and inorganic Zn as ZnO. Blood samples were collected at the start (d1) and at the end (d20A) of the experimental period before feeding and 3 hours after feeding (d20B) to determine plasma Zn and serum metallothionein (MT) concentration. Faecal samples were collected rectally, alternately in the morning (d15, 17, and 19) and afternoon (d16, 18, and 20) directly after feeding to calculate apparent nutrient digestibility and apparent Zn absorption. Neither Zn nor protein source affected Zn status (plasma Zn: P=0.288 and P=0.237, respectively, Serum MT: P=0.161 and P=0.193, respectively) or apparent Zn absorption (P=0.360 and P=0.527, respectively). Hydrolysed feather meal showed lower crude protein, crude fat, and crude ash digestibility compared to soybean meal (P<0.001). Faecal Zn concentration was not affected by Zn source (P=0.442). This study did not confirm the earlier observed *in vitro* effect of protein source on Zn bioavailability and shows that, at adequate levels commonly used in practice, the choice of Zn or protein source does not influence Zn status.

Introduction

Zinc, a micromineral, is important for a number of biochemical processes such as enzyme function, protein synthesis, hormone regulation, bone mineralisation, cell growth and differentiation, cell mediated immunity, and gene expression (McDowell, 2003; Lowe *et al.,* 2009). To assure normal biochemical processes, Zn is tightly regulated by the processes of absorption and (endogenous) excretion to maintain homeostasis (King *et al.,* 2000; McDowell, 2003; Hill and Link, 2009). Zinc absorption in the intestines is claimed to be influenced by the amount and source of Zn in the diet and by the interaction with other nutrients (Ammerman *et al.,* 1995; McDowell, 2003; Suttle, 2010). In practice, Zn is usually added to the diet at levels near the European legal maximum allowance (150 mg Zn/kg diet). Adding Zn above the animal's requirements (100 mg Zn/kg diet; NRC, 2012) might negatively influence the environment (soil and water conditions), if animal excretion of excessive Zn is continuously applied to the soil in excess of crop requirements (Jongbloed and Lenis, 1998; Revy *et al.,* 2004; Jongbloed, 2010). A possible strategy to reduce Zn excretion to the environment is to use Zn sources that are more easily absorbed by the animal, resulting in lower Zn supplementation (Spears, 1996; Jongbloed, 2010; Paulicks *et al.,* 2011).

Organic Zn sources are claimed to have a higher bioavailability[1] compared to inorganic Zn sources. However, the underlying mechanisms are not well understood (Spears, 1996; Wright and Spears, 2004) and results from previous research are inconsistent (Jongbloed, 2010; Paulicks *et al.,* 2011; Nitrayova *et al.,* 2012).

Moreover, the Zn requirements of breeding sows and the effect of other nutrients on Zn absorption such as proteins have not been fully determined. Previous *in vitro* research results demonstrated the possibility of spontaneous chelation of Zn in the presence of easily digestible protein sources (Van paemel and Janssens, 2008), which was also observed with calcium (Ca), copper (Cu), and manganese (Mn) (Zhu *et al.,* 2013). Spontaneous chelation may enhance Zn absorption. We therefore hypothesised that the protein source, and its concomitant profile of amino acid release during digestion, can affect the level of spontaneous chelation with Zn, thus altering the bioavailability of Zn. The present study investigated the possible interaction between Zn source and protein source on the *in vivo* Zn bioavailability in sows during late gestation fed adequate dietary Zn concentrations (*i.e.* above estimated requirements and below legal maximum allowance).

[1] Bioavailability is defined as the proportion of a nutrient capable of being absorbed and available for use or storage (Heaney, 2001; Srinivasan, 2001).

Materials and methods

Animals and management

Fifty-seven sows (RA-SE Genetics) from three succeeding groups in a 3-week interval from the experimental herd of the Institute for Agricultural and Fisheries Research (ILVO) were selected during late gestation (n = 57: 55 gravid sows at 86 days ± 1.3; 1 non-gravid sow, and 1 gravid sow at 20 days at the start of the experiment). The total duration of the experiment was 60 days, 20 days for each sow group. Per sow group, all sows were randomly divided into four dietary treatment groups. One multiparous sow aborted during the experiment and was subsequently excluded. Consequently, the experiment included 56 sows (parity: 3.7 ± 2.5). Parity was equally distributed among dietary treatment groups (parity per dietary treatment group: 3.5 ± 2.7 (n = 13), 3.7 ± 2.4 (n = 14), 4.0 ± 2.8 (n = 15), and 3.5 ± 2.4 (n = 14)). Sows' average bodyweight at the start of the study was 250 ± 43 kg. These sows were selected during late gestation, because previous results showed low plasma Zn concentrations representing Zn status during late gestation (van Riet *et al.,* 2015), indicating that these sows were challenged to maintain Zn homeostasis and may therefore absorb Zn more efficiently compared to other phases of the reproductive cycle.

The sows were housed in groups in free access stalls (maximum eight sows per compartment, three compartments). In each compartment, sows from the four treatment groups were present. The stalls were naturally ventilated and the surface area per sow was 2.78 m^2, including 1.17 m^2 for the individual stalls.

During feeding and faecal sampling sows were separated and the feeding stalls were locked. Each sow was randomly assigned to one of four experimental gestation diets throughout a 20-day experimental period. The feed allowance was 2.6 kg, provided in two equal portions at 8:00 a.m. and 2:30 p.m. Feed leftovers were collected after feeding and recorded per sow at the end of each week. Drinking water was provided automatically through individual nipple drinkers in the feeding troughs for 15 min every hour to avoid water spillage. One drink nipple per compartment opposite of the stalls ensured *ad libitum* access to water. One hour before feeding, water provision in the individual feeding troughs was suspended to facilitate cleaning. Water was provided again 30 min after feeding and after removing feed leftovers.

All sows were fed a gestation diet according to commercial dietary standards and nutrient requirements for gestating sows (NRC, 2012). A pre-experimental diet was fed for at least 3 weeks and contained 880.5 g/kg DM, 144.9 g/kg crude protein, 137 mg/kg Zn (originating from ingredients and Zn added as ZnO), and 5.2 g ileal digestible lysine per kg diet.

Dietary treatment

The treatment groups differed in the combination of Zn and protein source used in their gestation diet (provided between d84 and 108 of gestation) (Tables 5.1 and 5.2), yielding four possible combinations: 1) organic Zn + soybean meal, 2) inorganic Zn + soybean meal, 3) organic Zn + hydrolysed feather meal, and 4) inorganic Zn + hydrolysed feather meal.

Inorganic Zn was added as ZnO (75% Zn) (133 g ZnO per 1000 kg feed, INVE Belgium N.V., Baasrode, Belgium), and organic Zn as Availa®Zn containing Zn (10%) in an amino acid complex: single amino acids from hydrolysed soy proteins (molar ratio 1:1, 1000 g Availa®Zn per 1000 kg feed, Zinpro Corporation, Eden Prairie, MN, USA) to add 100 mg Zn/kg feed to the diets. The protein sources were soybean meal (44.5% CP) or hydrolysed feather meal (85% CP) that differ in their digestibility and were chosen to differentiate accordingly. To calculate nutrient digestibility, 0.75% acid insoluble ash (Celite 545, VWR International, Leuven, Belgium) was added as an indigestible marker. The diets were formulated to similar amino acid concentrations and net energy content.

All experimental procedures involving these sows were approved by ILVO's ethical committee (approval no. 2012/183, September 4th, 2012).

Measurements

The sows' body temperature was determined daily to monitor their health status. Feed leftovers were collected after feeding and recorded weekly; reproduction performances were obtained after weaning. Cross-fostering and provision of creep feed (transitional feed) to the piglets from day 10 postpartum simulated conventional conditions and could not be corrected for in the analysis. The reproductive performances are therefore interrelated to these conditions.

Table 5.1. Ingredient composition of the soybean meal and hydrolysed feather meal gestation diets.

Ingredients (g/kg fresh matter)	Soybean meal	Hydrolysed feather meal
Maize	250	250
Barley	143	150
Wheat bran	118	150
Wheat	110	111
Soybean meal	105	-
Beet pulp	100	100
Alfalfa meal	76	78
Hydrolysed feather meal	-	55
Beet molasses	30	36
Premix 3%*	30	30
Lard	24	24
Celite	7.5	7.5
Sodium bicarbonate	-	4.5
Salt	3.6	-
Limestone	0.6	-
L-Lysine HCL	0.6	3.0
L-Threonine	0.7	0.7
DL-Methionine	1.0	0.5
L-Tryptophan	0.1	0.4

* Premix 3% without Zn included per kg diet: vitamin A (12499 IU), vitamin D3 (1995 IU), vitamin E (60 mg), vitamin K3 (2.0 mg), vitamin B1 (2.0 mg), vitamin B2 (5.0 mg), vitamin B5 (20 mg), vitamin B6 (4.0 mg), vitamin B12 (0.04 mg), vitamin B3 (35 mg), vitamin B11 (3.0 mg), biotin (0.4 mg), choline (282 mg), $C_5H_{14}CINO$ (325 mg), $FeSO_4*H_2O$ (Fe: 80 mg/kg), $CuSO_4*5H_2O$ (Cu: 10 mg/kg), MnO (Mn: 80 mg/kg), anhydrous $Ca(IO_3)_2$ (I: 2 mg/kg), Na_2O_3Se (Se: 0.4 mg/kg), Ca (5.3 g), P (0.3 g), Mg (0.2 g), Na (1.5 g), Cl (2.8 g), K (0.1 g), Lysine (1.7 g), Methionine (0.02 g), Methionine +cysteine (0.04 g), Threonine (0.5 g), Tryptophan (0.02 g), Isoleucine (0.04 g), Histidine (0.03 g), Leucine (0.07 g), Valine (0.06 g), Arginine (0.07 g), 3-phytase (1000 FYT), linoleic acid (0.1 g), linolenic acid (0.01 g), anhydrous trimethylglycine (275 mg), Sepiolite (470 mg/kg), Bentonite-montmorillonite (470 mg/kg), formic acid (5.2 mg/kg), propionic acid (49 mg/kg), citric acid (1.5 mg/kg), Ethoxyquine (2.4 mg/kg), Butylated hydroxy anisol (1.9 mg/kg).

Table 5.2. Analysed and calculated nutrient composition of the soybean meal and hydrolysed feather meal with organic or inorganic Zn source gestation diets.

Chemical analysis (g/kg)*	SB		HF	
	Organic	Inorganic	Organic	Inorganic
Dry matter	886	887	887	887
Crude ash	64	64	59	59
Crude protein	145	143	148	143
Crude fat	51	49	51	51
Zn (mg/kg)	135	124	146	141
Starch	305		313	
Sugar	52		45	
ADF	88		85	
NDF	199		203	
ADL	15		16	
Ca	8.5		8.5	
P	4.2		4.2	
Cu (mg/kg)	18		18	
ID Lysine	6.6		6.6	
ID Methionine	2.7		1.9	
ID Methionine+ cystine	4.3		4.5	
ID Threonine	4.3		4.3	
ID Tryptophan	1.2		1.2	
ID Arginine	6.7		6.4	
ID Leucine	8.2		8.3	
ID Isoleucine	3.9		4.1	
ID Histidine	2.8		2.0	
ID Valine	4.6		5.6	
ID Phenylalaline	4.9		4.8	
NEv (MJ/kg)	9.0		9.0	

SB, gestation diet with soybean meal as protein source; HF, gestation diet with hydrolysed feather meal as protein source; ID, ileal digestible; NEv, net energy for pigs.

* Chemical analyses of starch, sugar, ADF, NDF, ADL, Ca, P, Cu, the ID amino acids and NEv are calculated according to the feed tables of the Centraal Veevoederbureau (CVB, The Netherlands), 2007.

Bodyweight and backfat thickness

Bodyweight and backfat thickness were measured at the beginning and ending of the experimental period for each group. Backfat thickness was included to monitor the condition of sows, which fluctuate according to reproductive phase. Backfat thickness was determined between the 3rd and 4th last rib, 7 cm from the left and right side of the vertebrae (P2 position). After P2 was lubricated,

backfat measurements were determined alternately at the left and right sides (Renco Lean Meater-12 60566, Renco Corporation, Minneapolis, MN, USA). If the difference between left and right was 2 mm or more, the measurements were repeated up to three times. The average backfat thickness along with bodyweight was used to determine if the sows' condition was influenced by dietary treatment.

Blood sampling and biomarker analyses

Blood samples (20 mL) were taken from all sows at the start (d1) and end (d20A) of the experiment before feeding in the morning (at 8.30 h AM), after overnight fasting (18 hours). On d20, blood samples were also collected 3 hours after the morning feeding (d20B). Blood samples were collected from the jugular vein using stainless steel needles and plastic syringes, and added to one heparin and one serum vacuum tube (Terumo Europe, Leuven, Belgium).

One millilitre heparinised blood was used to determine haematocrit (centrifuged 2749 *g*, 30 min, 20 ºC) to monitor health status of the sows. The remainder was centrifuged (1500 *g*, 10 min, 4 ºC) and plasma was divided over two 5-mL disposable polystyrene tubes. Tubes were stored for 24 hours at -20 ºC, and then transferred to storage at -80 ºC until analysis of plasma Zn. The vacuum serum tubes were centrifuged (1500 g, 10 min, 4 °C) after resting overnight at 4 °C to allow clotting. Serum samples were equally divided between two 5-ml disposable polystyrene tubes, stored for 24 h at -20 °C and then at -80 °C until analysis of serum metallothionein (MT).

For plasma Zn analysis, a commercial kit was used (Randox kit, ZN2341, Randox Laboratories Ltd., Crumlin, UK). First, plasma samples of d0, 20A, and 20B were deproteinated (Randox ZN2607, Randox Laboratories Ltd., Crumlin, UK) by mixing them with an equal volume of trichloroacetic acid and centrifuging for 10 min at 10,000 *g*. The remaining supernatant was used within 2 hours to determine plasma Zn concentrations. The deproteinated plasma was diluted 5 times with a colour reagent, and incubated for 5 min at 25 ºC. The absorbance was measured at a wavelength of 570 nm with a reference wavelength of 620 nm using a microplate reader (EZ reader 400, Biochrom Ltd., Cambridge, UK). The plasma Zn concentration was interpolated from the multipoint standard calibration curve. The inter- and intra assay coefficients of variability were 2.19 and 3.61 %, respectively. The minimum and maximum recovery was 99.5 and 119.8 %, respectively (van Riet *et al.*, 2015).

Serum metallothionein (MT) was determined at d0 and d20A using competitive ELISA (Porcine Metallothionein (MET) Elisa kit, E07M0030, BlueGene Biotech CO., Shanghai, China). The product of the enzyme-substrate reaction forms a blue coloured complex that turns yellow after a stop solution was added to stop the reaction. The intensity of the colour was measured

spectrophotometrically at 450 nm (BEP 2000, Siemens AG, Munich, Germany). The MT concentration was interpolated from the standard curve.

Nutrient digestibility

Faecal samples were collected rectally during 6 days after a dietary adaptation period of 14 days. Samples were collected in the morning (d15, 17, and 19) and afternoon (d16, 18, and 20) of alternate days, directly after feeding. The faecal samples were collected manually using a lubricated glove then weighed and stored at -20 ºC in a plastic bag. Samples from each sampling moment per sow were stored separately. The average amount of faeces collected was 192.8± 124 gram (mean± SD).

Per sow, the frozen samples (morning or afternoon) were thawed in the plastic bag for 24 hours in a closed bucket to prevent dehydration. After thawing, the 3 morning samples were pooled; likewise the 3 afternoon samples. Pooled samples were mixed for at least 1 min, with a weighed quantity of demineralised water added as needed to soften faeces (Zn concentration analysed: <0.020 mg/l). Representative homogenised faecal samples were collected for nitrogen determination (250 g) to calculate protein concentration (ISO 5983-2, 2005), and >200 g was placed in a Petri dish, weighed, then stored again at -20 ºC for lyophilisation. After lyophilisation, the pooled samples were ground to pass through a 1 mm sieve for nutrient digestibility calculations. A fraction of at least 2.5 g was further ground to 0.5 mm to analyse Zn concentration.

The ground feed and faecal samples were subjected to proximate analysis (ISO 17025, 2005) to determine the crude protein, crude fat, crude ash, and acid insoluble ash (AIA) fraction. The apparent nutrient digestibility coefficients (g/g) and apparent Zn absorption of faecal samples (g/g) was calculated using the formula:

$$(1 - ((Nutrient_{faeces}/Nutrient_{feed})*(AIA_{feed}/AIA_{faeces})))$$

The Zn concentration in feed and faeces was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Vista MPX, Varian Inc., Palo Alto, CA, USA) after microwave-assisted matrix digestion with HNO_3 and H_2O_2.

Statistical analysis

Performance characteristics (bodyweight and backfat thickness) were analysed using a linear mixed model with day (d1-20A), Zn source, protein source, the interaction between Zn and protein source, and parity as fixed effects. Sow and group were added as random effects to correct for repeated

measures within sows and groups. Reproductive performances were analysed using a linear mixed model for bodyweight of the piglets at birth and weaning with Zn source, protein source, the interaction between Zn and protein source, and parity as fixed effects and group as random effect to correct for repeated measures within group. A similar Poisson mixed model was used for the performance characteristics related to the number of born piglets. The interaction between Zn and protein source was excluded from the final models if not significant.

Blood biomarkers (plasma Zn and serum MT concentrations) were analysed using a linear mixed model with day (d1-20A) or part of the day (d20A-20B for plasma Zn), Zn source, protein source, the interaction between Zn and protein source, and parity as fixed effects. Sow and group were added as random effects to correct for repeated measures within sows and groups. Observations for serum MT were log-transformed to obtain a normal distribution of the residuals of the model. In a separate linear mixed model, plasma Zn concentration as well as parity was used as fixed effects to detect associations between plasma Zn and serum MT concentrations.

The pooled morning and pooled afternoon faecal samples were averaged and used as outcome variable for nutrient digestibility, apparent Zn absorption, and faecal Zn concentration. These values were analysed using linear mixed models with Zn source, protein source, the interaction between Zn and protein source, and parity as fixed effects. Group was added as random effect to correct for repeated measures within group. The interaction between Zn and protein source was excluded from the final models if not significant. In a separate linear mixed model, the association between plasma Zn concentration and apparent Zn absorption was analysed with apparent Zn absorption as fixed effect.

The analysed data (serum MT after log transformation) were considered sufficiently normally distributed, based on the graphical evaluation (histogram and QQ-plot) of the residuals. All analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Performance

One primiparous and one multiparous sow aborted before the experiment. The primiparous sow was re-inseminated and was gravid for 20 days at the start of the experiment. Both sows were excluded from the statistical analyses. Two multiparous sows did not eat their full ration for several days. These sows remained in the experiment, because their results were not considered outliers compared to other sows. The results are presented as mean± SE.

Body temperature did not show any outliers. In total, 23 sows had feed leftovers throughout the experiment with an average amount of 1.3 ± 0.5 kg per sow. During the first 7 days, feed leftovers

yielded on average 0.7 ± 0.5 kg per sow (d1-7, n = 17). The number of sows with feed leftovers decreased to three during d8-14 and d15-20. The average amount of feed leftovers were then 4.7 ± 2.3 kg (d8-14, n = 3) and 1.4 ± 0.7 kg (d15-20 corresponding to the faecal collection period, n = 3).
The average bodyweight of piglets born alive was 1.5 ± 0.0 kg, average bodyweight of weaned piglets was 8.2 ± 0.1 kg, and the number of piglets born alive, number of stillborn piglets, and number of weaned piglets was 13 ± 0.5, 1.6 ± 0.3, and 10.5 ± 0.4, respectively. Piglet mortality (n) was 2.5 ± 0.3. No interaction was found between Zn and protein source. The organic Zn source fed to sows tended to lower the average bodyweight of piglets born alive (P=0.054) and piglet mortality was lower for sows fed hydrolysed feather meal (1.8± 0.3) compared to soybean meal (3.1± 0.4) (P=0.002).
Bodyweight increased from 251± 6 kg (d1) to 263± 6 kg (d20A; $P<0.001$), and neither Zn nor protein source influenced bodyweight changes ($P=0.697$ and $P=0.437$, respectively). Primiparous sows showed a significantly lower bodyweight compared to multiparous sows (194 and 268 kg, respectively, $P<0.001$).
Backfat thickness was not different between d1 (19± 0.4 mm) and d20A (20± 0.4 mm) ($P=0.141$) and was not associated with Zn source or protein source ($P=0.494$ and $P=0.732$, respectively). Primiparous sows had a significantly lower backfat thickness compared to multiparous sows (15 and 21 mm respectively, $P=0.013$).

Zinc status biomarkers

Haematocrit was 40± 1% at d1, 38± 0.4% at d20A, and 37± 0.5% 3 hours after feeding (d20B). This was not associated with plasma Zn concentrations.
Plasma Zn concentration tended to decrease towards d20A ($P=0.053$) (Figure 5.1). The variation in plasma Zn concentrations among sows was high. Neither Zn nor protein source were associated with plasma Zn concentrations ($P=0.288$ and $P=0.237$, respectively) nor did Zn and protein source interact ($P=0.158$). Furthermore, plasma Zn concentration was lower 3 hours after feeding on d20 (d20B) compared with before feeding (d20A) ($P=0.039$). A tendency for an interaction was found between Zn and protein source on plasma Zn concentration when d20A was compared with three hours after feeding (d20B) (P=0.056): hydrolysed feather meal and organic Zn showed higher plasma Zn concentrations.
Serum MT concentration increased from d1 towards 20 ($P<0.001$) (Figure 5.2). Zinc and protein source were not associated with these serum MT concentrations ($P=0.161$ and $P=0.193$), but plasma Zn concentration was ($P=0.045$).

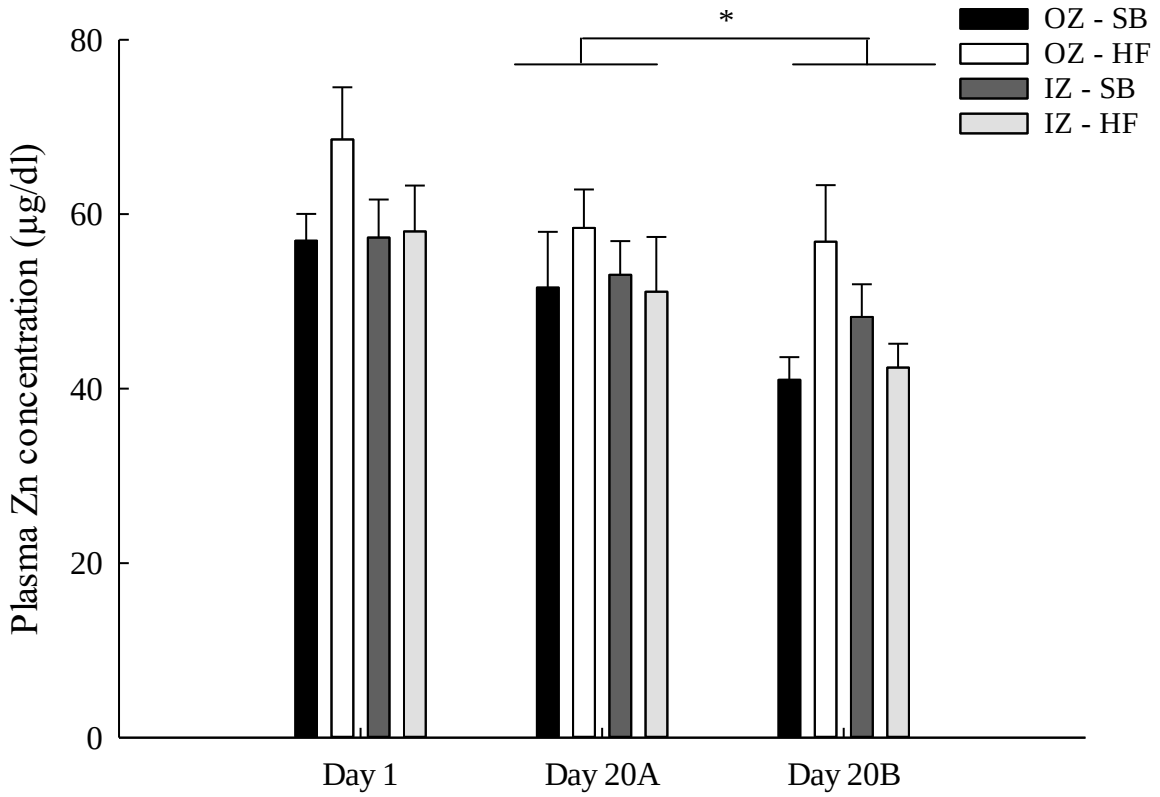

Figure 5.1. Plasma Zn concentrations (µg/dL) at the start of the experiment before feeding (d1) and after a 20-day experimental period before feeding (d20A) and 3 hours after feeding (d20B) of 3 succeeding groups of sows (n= 54). Values are means, with their standard errors represented by vertical bars. OZ= Organic Zn source, IZ= inorganic Zn source (ZnO), SB= Soybean meal, HF= Hydrolysed feather meal, * represents significant differences $P<0.050$. Plasma Zn concentration tended to decrease towards d20A ($P=0.053$) and was lower at d20B ($P=0.039$). Zinc source ($P=0.288$) and protein source ($P=0.237$) did not influence the plasma Zn concentration. A tendency for an interaction was found between Zn and protein source on plasma Zn concentration when d20A was compared with three hours after feeding (d20B) (P=0.056).

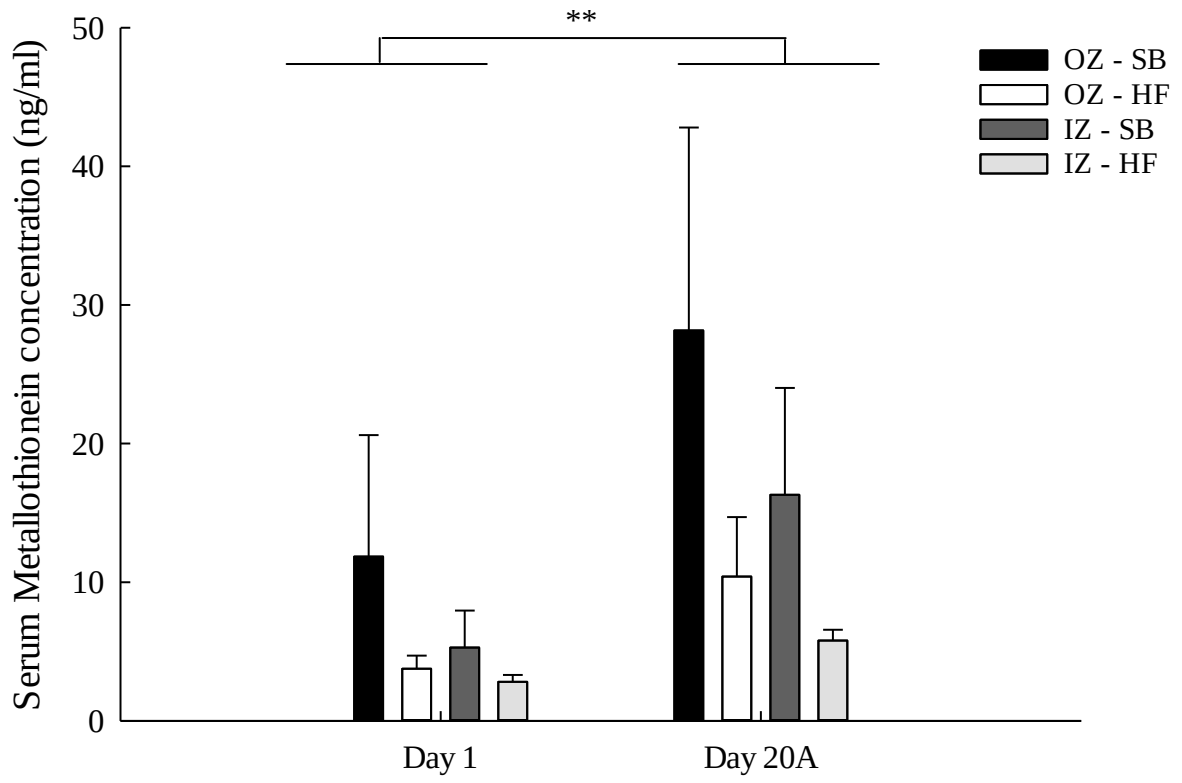

Figure 5.2. Serum metallothionein (MT) concentrations (ng/mL) at the start of the experiment, before feeding (d1) and after a 20-day experimental period before feeding (d20A) of 3 succeeding groups of sows (n= 54). Values are means, with their standard errors represented by vertical bars. OZ= organic Zn source, IZ= inorganic Zn source (ZnO), SB= Soybean meal, HF= Hydrolysed feather meal, ** represents significant differences $P<0.010$. Serum MT concentration increased towards d20A ($P<0.001$). Zinc source ($P=0.161$), protein source ($P=0.193$), and interaction between Zn and protein source were not different.

Nutrient digestibility

There was no significant effect of Zn or protein source on apparent Zn absorption (*P*=0.360 and *P*=0.527, respectively) (Table 5.3). A negative association was found between plasma Zn concentration and apparent Zn absorption (*P*=0.045).

Hydrolysed feather meal diets showed lower apparent faecal crude protein (*P*<0.001), crude fat (*P*<0.001), and crude ash (*P*<0.001) digestibility (Table 5.3). An interaction between Zn and protein source was found for crude protein digestibility (*P*=0.003): protein digestibility was higher for hydrolysed feather meal with organic Zn.

Primiparous sows (0.66± 0.7 g/g) showed a lower (*P*=0.003) crude fat digestibility compared to multiparous sows (0.69± 0.5 g/g) and tended to have a lower (*P*=0.050) crude protein digestibility compared to multiparous sows (0.75± 1.0 g/g and 0.76± 0.5 g/g, respectively).

Faecal Zn concentration was not different between organic and inorganic Zn source (*P*=0.442) and protein source was also not associated with these faecal Zn concentrations (*P*=0.385) (Table 5.3).

Table 5.3. Apparent nutrient digestibility coefficients, apparent Zn absorption, and faecal Zn concentration in sows during late gestation (n= 54).

	Zinc source		Protein Source			*P*		
Item*	Organic	Inorganic	SB	HF	SEM	Z	P	Z*P
Crude protein (g/g)	0.76	0.75	0.79	0.72	0.004	0.166	<0.001	0.003
Crude fat (g/g)	0.69	0.68	0.71	0.66	0.005	0.385	<0.001	NS
Crude ash (g/g)	0.40	0.41	0.44	0.37	0.005	0.194	<0.001	NS
Zn Abs (g/g)	0.05	0.04	0.04	0.05	0.007	0.360	0.527	NS
Faecal Zn (mg/kg)	214.1	209.9	214.4	209.8	2.6	0.442	0.385	NS

SB, Soybean meal; HF, Hydrolysed feather meal; Z, Zn source; P, Protein source; Z*P, interaction between Zn source and protein source. If not significantly different (NS, *P*>0.050) then excluded from the final models; Zn Abs, Apparent Zn absorption.

* Nutient digestibility is expressed as the coefficient of digestibility of the specific nutrients (g/g).

Discussion

Results of the present study demonstrate that neither Zn source nor protein source affected either Zn status or apparent Zn absorption in sows during late gestation.

Zinc bioavailability in pigs

Previous studies in pigs, mainly piglets and fattening pigs, have shown contradictory results on Zn bioavailability for several organic sources. Some studies found a higher bioavailability of organic Zn, whereas other studies did not (Revy *et al.*, 2002; Siebert *et al.*, 2010; Paulicks *et al.*, 2011). Zacharias *et al.* (2007) and Nitrayova *et al.* (2012) reported a positive effect of organic Zn on Zn

bioavailability. Zinc digestibility was higher (0.26 g/g) and Zn concentration in the faeces was lower (193.4 mg/d) for the organic Zn source in which Cu and Zn were chelated with partially hydrolysed soybean proteins compared with Zn sulphate (0.16 g/g and 214.5 mg/d, respectively) (Zacharias *et al.,* 2007). However, it seems that this effect was dependent on Zn inclusion level and dietary Zn concentration, because no effects on the parameters for bioavailability were found when 30 mg added Zn/kg diet was fed instead of 70 mg added Zn/kg diet. Nitrayova *et al.* (2012) tested several organic Zn sources against ZnO. In this study, 10 mg Zn/kg diet was added to 49.7 mg Zn/kg of the basal diet. Zinc was added as ZnO, Zn methionine, Zn glycine, Zn proteinate, and Zn yeast. The apparent Zn digestibility was lower for ZnO (0.17 g/g) compared with Zn methionine (0.52 g/g) and Zn retention lower for ZnO (16.4 % of Zn intake) compared with Zn methionine and Zn yeast (51.0 and 45.3% of Zn intake, respectively). Blood biomarkers, however, showed no differences between Zn sources (Nitrayova *et al.,* 2012). Therefore, the responses observed for the different parameters seem to be also affected by the parameters used to determine Zn bioavailability. This shows that the findings of these previous studies are equivocal and the underlying mechanism (specific processes) seems not to be fully understood (Spears, 1996; Wright and Spears, 2004). This is further supported by Jongbloed (2010): based on 16 studies, including 29 experiments, they concluded that a Zn source with improved bioavailability over inorganic Zn sources could not be found. The dietary Zn concentration of the studies included was close or above estimated Zn requirements and the bioavailability ranged between 0.84 and 1.20 g/g irrespective of dietary Zn concentrations (Jongbloed, 2010).

Studies on Zn bioavailability in sows are scarce and focus on reproductive performance (Jongbloed *et al.,* 2010; Bikker and Jongbloed, 2014), showing beneficial effects of added dietary microminerals (Payne *et al.,* 2006; Peters and Mahan, 2008; Anil, 2011). No effect of a combined organic micromineral supplement (Zn, Fe, Cu, and Mn) on reproductive performance was found in another study, except for the interaction between Zn source and dietary Zn concentration for average weaning weight (Acda and Chae, 2002). Although the present *in vivo* study was not designed to evaluate the effect of Zn and protein source on reproductive performance, Zn source tended to influence average bodyweight of piglets born alive and protein source did influence piglet mortality. The condition of sows was not influenced by Zn source.

Factors besides the influence of Zn inclusion level, dietary Zn concentration and response markers chosen based on the reported bioavailability in other studies may also be related to the discrepancies found in literature. These factors include chelation effectiveness of the organic Zn source (*e.g.* ligand used to form the complex or chelate) versus chelation effectiveness of the non-digestible diet fractions (Cao *et al.,* 2000; Spears *et al.,* 2004; Nitrayova *et al.,* 2012), solubility (Cao *et al.,* 2000;

de Souza *et al.*, 2007; Nitrayova *et al.*, 2012), Zn status of animals (*e.g.* Zn depleted, Zn deficient, or adequate Zn status), presence of stress (Nockels *et al.*, 1993; Kellogg *et al.*, 2004), inorganic Zn source used as control (*e.g.* mostly sulphates), and the composition of the basal diets (*e.g.* corn-soybean, semi-synthetic or wheat/barley diets, addition of phytase) (Revy *et al.*, 2004; Bikker *et al.*, 2011; Bikker and Jongbloed, 2014).

Spontaneous chelation

Proteination supposedly improves bioavailability of the mineral to target cells and organs important for Zn metabolism (Rompala and Halley, 1995; Anil, 2011). A previous *in vitro* study showed the possibility of spontaneous chelation of Zn when easily digestible protein sources were present (van Paemel and Janssens, 2008). However, the present *in vivo* study does not support these findings: neither Zn nor protein source affected apparent zinc absorption. The proximal protein digestibility might not have differed enough between the selected protein sources; also, an effect might not have been detected with the techniques used in the present study. Additionally, the markers used to determine Zn bioavailability, differences in dietary phytate content, interactions between Zn and other minerals, the sows' body condition at late gestation, and the potentially increased endogenous Zn loss in the presence of increased absorption may also explain our results. Our attempt to use ultracentrifugation as a method to quantify the soluble Zn fraction representing the endogenous Zn fraction excreted in the faeces was unsuccessful (results not shown).

The organic Zn source in the present study improved the protein digestibility for hydrolysed feather meal. To our knowledge, this has not been reported previously. The effect observed may have been co-incidental or due to methodological constraints, but without speculating on the exact mechanism, the higher protein digestibility of soybean meal might have overruled any effect of Zn source on protein digestibility. Further studies should clarify whether Zn source can affect protein digestibility in general, or whether this effect arises because of a feature typical of hydrolysed feather meal.

Zinc status

Plasma Zn and serum MT concentration are biochemical markers for Zn status and plasma Zn concentration is (besides bone Zn concentration) most often used in bioavailability studies in pigs if the Zn supply is ≤250 mg/kg. However, Zn source did not affect the observed concentrations in sows although the dietary Zn concentration was below 250 mg/kg. Similar results were found in other studies in pigs for plasma Zn concentration showing no effect of Zn source (Swinkels *et al.*, 1996; Revy *et al.*, 2002, 2004; Schlegel *et al.*, 2010). Jongbloed (2010) suggested that the animal category (*e.g.* piglets, fattening pigs, sows) influences the observed responses.

The lower plasma Zn concentration at three hours after feeding (d20B) is probably a result of the circadian rhythm of Zn and not to an altered absorption of Zn. In a previous study in sows, plasma Zn decreased throughout the day (van Riet *et al.*, 2014), similar to the pattern observed in humans (Markowitz *et al.*, 1985; Hambidge *et al.*, 1989; King *et al.*, 1994). If Zn was better absorbed in the present study, plasma Zn concentration should have been increased, because Zn is transported after absorption in the plasma predominantly bound to albumin and transported to the liver and incorporated in other tissues (Hess *et al.*, 2007; Gibson *et al.*, 2008; Naithani *et al.*, 2014).

Zinc source and its effect on serum MT concentrations have not been reported in previous studies. In the present study, serum MT concentrations increased over time, opposite from the tendency to decreased plasma Zn concentrations. This difference in change over time may be indicative for redistributing tissue Zn (low plasma Zn and high serum MT concentrations) rather than a reduction in the readily exchangeable Zn pool (King, 1990). Reductions in the readily exchangeable Zn pool are an indication of suboptimal Zn status and thus lower Zn absorption (low plasma Zn and low serum MT concentrations) as found in humans (King, 1990; Gibson *et al.*, 2008). Redistribution of tissue Zn may be caused by confounding factors such as subclinical infection, stress, or hormonal changes. Infections were not observed throughout the experimental period of the present study and may not be the cause for the increased serum MT concentrations. The opposite behaviour of serum MT concentration and plasma Zn concentration indicate the need for caution when interpreting these biomarkers for bioavailability measurements.

Zinc excretion

The strategy to use more bioavailable Zn sources to reduce Zn excretion to the environment (Jongbloed, 2010; Paulicks *et al.*, 2011) is not supported by the present study: faecal Zn concentration was not different between organic and inorganic Zn sources. This supports the assumption that it is dietary Zn concentration rather than Zn source that mainly determines Zn excretion (Case and Carlson, 2002; Carlson *et al.*, 2004).

Conclusion

In the present study, neither Zn nor protein source affected either Zn status or apparent Zn absorption in sows during late gestation and no interaction was observed between Zn and protein source. This study did not confirm the previously reported *in vitro* effect of protein source on Zn bioavailability when evaluated in sows fed Zn above their requirements. Faecal Zn concentration was not affected by Zn source.

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Chapter 6

Dietary zinc concentration and claw quality

Chapter 6a

Marginal dietary zinc concentration and claw quality in weaned piglets

Adapted from: Marginal dietary zinc concentration affects claw conformation measurements but not histological claw characteristics in weaned piglets.

M.M.J. van Riet, G.P.J. Janssens, P. Cornillie, W. Van Den Broeck, E. Nalon, B. Ampe, F.A.M. Tuyttens, D. Maes, G. Du Laing, S. Millet.

Submitted

Abstract

The objective of the present study was to explore whether marginal dietary zinc (Zn) concentration affects claw quality measurements in weaned piglets. Twenty-four weaned piglets were randomly assigned to two dietary treatment groups: 42 mg Zn/kg diet from ingredients only (unsupplemented, marginal dietary Zn concentration below Zn requirements of 80 mg Zn/kg feed), and 106 mg Zn/kg diet, where Zn was added as ZnO (conventional dietary Zn concentration). Claw conformation characteristics were measured at the start (d0, 4 weeks of age) and at the end (d36) of the study and the histological claw characteristics of horn wall and heel horn were examined on samples collected at 9 weeks of age.

Non-supplemented piglets had a narrower claw width (P=0.028), and lower toe heights (P=0.010) at 9 weeks. The length of the dorsal border tended to be lower for the non-supplemented piglets (P=0.092). Claw volume and claw horn size were smaller (P=0.003 and P<0.001, respectively) for the non-supplemented piglets at nine weeks. Horn growth and wear were lower for the non-supplemented piglets (P=0.044 and P<0.001, respectively), but net horn growth (horn growth minus wear) was not different (P=0.406). No changes in histological claw characteristics were observed. Differences in claw quality measurements were found between lateral and medial claw digits and between front and hind claws.

Marginal dietary Zn concentration seems to affect various claw quality measurements, but initial differences in claw conformation may have been (partly) interfered with the results observed at the end of the study. Though, several measurements indicated that these marginal dietary Zn concentrations may not be sufficient to maintain claw quality in piglets.

Introduction

Claw quality is determined as the product of horn characteristics to support the inner structure of the digits and assist in the dispersal of weight and stress during locomotion (Vermunt and Greenough, 1995; Lethbridge, 2009). It is evaluated by visual scoring for claw shape, claw shape dimensions, claw scoring, and measurement of structural, physical and biochemical properties of the claw horn (Politiek *et al.,* 1986; Vermunt and Greenough, 1995). The claw quality of pigs depends largely on the quality of horn production, which is influenced by the nutrient supply to the avascular epidermis (Tomlinson *et al.,* 2004; Muelling, 2009; Torrison, 2010). Poor horn quality can lead to development of claw lesions, such as separations along the white line, haemorrhages and cracks at the horn wall. Lower horn quality may be a result of trauma or impaired horn production, along with excessive or inadequate wear (Ossent, 2010, Torrison, 2010). Lameness is evident in 5-20 % of sows with claw lesions (Anil *et al.,* 2007) and they have considerable economic and welfare consequences (Heinonen *et al.,* 2013).

Nutrition is an important factor in impaired horn production, especially if the nutrient supply of for example amino acids, vitamins and minerals is insufficient (Butler and Hintz, 1977; Tomlinson *et al.,* 2004; Muelling, 2009). This causes perturbation of nutrient diffusion from the dermis to the avascular epidermis, thereby reducing claw quality, which in turn increases the susceptibility to damage from the environment (Tomlinson *et al.,* 2004; Muelling, 2009). The catalytic, structural, and regulatory functions of zinc (Zn) may all influence the processes required for horn production (Tomlinson *et al.,* 2004; Andrieu, 2008; van Riet *et al.,* 2013).

Despite the demonstrated effect of Zn on claw quality in previous studies in cows (Moore *et al.,* 1988; Enjalbert *et al.,* 2006; Nocek *et al.,* 2000 and 2006), only a few studies have been performed in pigs evaluating differences between Zn sources (Anil, 2011; Bradley, 2010). The pig industry has previously focused on intensifying body growth with less priority given to leg and claw conformation (Kroneman *et al.,* 1992). Nevertheless, claw lesions and lameness are an increasing economic and welfare concern, especially in sows (Anil *et al.,* 2005; Heinonen *et al.,* 2013). In the present study, piglets were used to evaluate if Zn is important in claw development and as an animal model to develop required methodologies. Moreover, the maximum dietary Zn allowances for weaned piglets in Europe to minimise Zn excretion to the environment is 150 mg Zn/kg, although reported Zn requirements to avoid Zn deficiency are lower (80 mg Zn/kg) (McDowell, 2003; Van paemel *et al.,* 2010; EFSA, 2014). Horn is continuously produced and requires Zn (Vermunt and Greenough, 1995; Winkler, 2005); it is therefore worth questioning whether a marginal dietary Zn supply is sufficient to maintain claw quality.

The aim of the present study was to explore whether a five-week period of marginal dietary Zn provision (*i.e.* below Zn requirements) results in observable claw quality differences, assessed by several measurements for claw conformation and histological claw characteristics.

Materials and methods

Animals, housing, and dietary treatment

Twenty-four weaned piglets (Piétrain boar * hybrid sow, 27.8± 1.6 days old) from the herd at the Institute for Agricultural and Fisheries Research (ILVO), bodyweight at weaning of 9.3± 0.3 kg, were selected for homogeneous groups and allocated randomly to one of two treatment groups (n= 12 piglets per treatment). Each pen, comprising one treatment group, held six non-sibling piglets (three barrows and three sows). The piglets were vaccinated against mycoplasma (Ingelvac Mycoflex, Boehringer Ingelheim) around 11 days postpartum and against Porcine Circovirus type 2 (Ingelvac Circoflex, Boehringer Ingelheim) around 19 days postpartum.

The piglets were housed in a conventional pen (floor area 205 cm × 180 cm) with a plastic slatted floor, a feeding trough along the full length of the pen, and two drinking nipples at the opposite sides of the feeding trough. Two stainless steel chains per pen were provided as environmental enrichment.

All piglets were fed a meal diet according to commercial standards and met the nutrient requirements for piglets (NRC, 1998) (Table 1 and 2). To prevent diarrhoea, colistin (1 g/piglet) was added to the diet for five days after weaning, and after a two-day break, it was again provided for five days. The non-supplemented piglets of the control group (n= 12 piglets) received 42 mg Zn/kg diet, originating from the ingredients only (marginal dietary Zn concentration below Zn requirements). The Zn-supplemented piglets (n= 12 piglets) received in total 106 mg Zn/kg diet (analysed), in which Zn was added as ZnO via premix (adequate dietary Zn concentration; median Zn concentration in conventional diets is 137 mg Zn/kg, EFSA, 2014). This dosage was chosen based on commercial diets. Water and feed were provided ad libitum. Feed samples were subject to proximate analysis (crude nutrient analysis) according to international standard methods accredited by ISO 17025 (2005) and performed by the accredited laboratory of the Animal Sciences Unit of ILVO. Dry matter (DM) content was determined using the procedure 71/393/EEC. Other procedures were ISO 5984 (crude ash), ISO 5983-2 (crude protein), AOCS approved procedure Ba 6a-05 (crude fibre), ISO 6492 (crude fat), Van Soest *et al.* (1991) (ADF, NDF, ADL), ISO 6490/1 (Ca), and ISO 6491 (P).

Table 6.1. Ingredient composition of the meal diet fed to weaned piglets with and without Zn supplemention (n= 24 piglets) during a 5-week experimental period.

Ingredients (g/kg fresh matter)	Meal diet
Barley	258.2
Maize	186.6
Wheat	174.8
Soybean meal	150.0
Premix 6 % without Zn*	60.0
Soybeans heated	58.1
Starpro 40†	40.0
Beet molasses	30.0
Soy oil	14.3
Nutrisure‡	10,0
Limestone	3.8
Monocalcium phosphate	1.7
Salt	1.7
L-Lysine HCL	4.4
L-Threonine	2.0
L-Valine	1.1
DL-Methionine	2.1
L-Tryptophan	0.8
Phytase	0.1

* Premix 6 % without Zn, included per kg diet: vitamin A (15000 IU), vitamin D3 (2000 IU), vitamin E (100 mg), vitamin K3 (20 mg), vitamin B1 (2.5 mg), vitamin B2 (7.5 mg), vitamin B5 (20 mg), vitamin B6 (5 mg), vitamin B12 (0.04 mg), vitamin C (100 mg), vitamin B3 (30 mg), vitamin B11 (3 mg), biotin (0.2 mg), choline (324 mg), $FeSO_4*H_2O$ (Fe: 100 mg), $CuSO_4*5H_2O$ (Cu: 160 mg), $MnSO_4*H_2O$ (Mn: 60 mg), $Ca(IO_3)_2$ (I: 2 mg), Na_2O_3Se (Se: 0.4 mg), Ca (483 mg), P (423 mg), Mg (165 mg), Na (326 mg), Cl (1514 mg), K (1183 mg), S (235 mg), Lysine (341 mg), Methionine (77 mg), Threonine (227 mg), Tryptophan (68 mg), Butylhydroxytoluene (13 mg), Ethoxyquine (13 mg), Propyl gallate (3 mg), Citric acid (13 mg).
† Protein concentrate (DSM nutritional products, Basel, Switzerland).
‡ DSM nutritional products: A mixture of calcium salts of the following organic acids: lactic acid, formic acid, citric acid monohydrate, orthophosphoric acid, propionic acid.

Table 6.2. Analysed nutrient composition of the meal diet fed to weaned piglets with and without Zn supplemention (n= 24 piglets) during a 5-week experimental period.

Chemical analysis (g/kg)*	Meal diet
Dry matter	896.8
Crude ash	48.5
Crude protein	196.0
Crude fibre	27.7
Crude fat	58.2
Starch	362.7
Sugar	78.8
ADF	34.9
NDF	90.7
ADL	2.4
Ca	6.3
P	5.3
Cu (mg/kg)	159.0
Zn (mg/kg)†	41.9/106.0
ID Lysine	11.5
ID Methionine	4.9
ID Threonine	7.2
ID Tryptophan	2.5
ID Arginine	9.4
ID Leucine	11.4
ID Isoleucine	6.0
ID Histidine	3.7
ID Valine	7.8
ID Phenylalaline	7.3
NEv (MJ/kg)	9.8

ADF, acid detergent fibre; NDF, neutral detergent fibre; ADL, acid detergent lignin; ID, ileal digestible; NEv, net energy for pigs.

* Chemical analyses of the apparent ID amino acids and NEv are calculated according to the feed tables of the Centraal Veevoederbureau (CVB, The Netherlands), 2007.

† The Zn concentration was 41.9 mg/kg for the non-supplemented diet (marginal dietary Zn concentration, below Zn requirements) and 106.0 mg/kg for the Zn-supplemented diet (Zn added as ZnO).

All experimental procedures were approved by ILVO's ethical committee (approval no. 2012/174, February 17th, 2012).

Samples and Measurements

Bodyweight was measured at 4 (d0, weaning; start of the study), 6 and 9 (d36, end of experiment) weeks of age.

After this 5-week period the piglets were sedated before euthanasia using an intramuscular injection of 7 mL/piglet mixture of Xyl-M 2 % (20 mg xylazine per mL; VMD N.V.) and Zoletil 100 (250 mg Tiletamine base and 250 mg Zolazepam base; Virbac S.A.). Consequently, blood was collected after sedation via cardiac puncture (20 mL), because piglets were euthanased after blood collection and claw dimension measurements. Blood was analysed for haematocrit percentage, plasma Zn and copper (Cu) concentration.

Claw conformation, expressed as dimensions, was determined using a digital calliper (Mitutoyo Belgium N.V.) at 4 weeks (d0) and at 9 weeks of age (d36) by the same observer. The claw dimensions (Figure 6.1) included sole (base) length, claw width, length of the dorsal border, diagonal claw length, toe height, heel height, and claw length, following a methodology adapted from Calabotta *et al.* (1982) and Vermunt and Greenough (1995). These dimensions were subsequently used to calculate the distal toe angle (sine of the length of the dorsal border and toe height), sole area (claw length × claw width), claw volume (sole area × heel height), claw horn size (claw width × diagonal claw length), and toe:heel ratio (toe height : heel height), representing nominal dimensions (Calabotta *et al.*, 1982; Vermunt and Greenough, 1995; Manske, 2002; Bradley, 2008; Van Amstel and Doherty, 2010).

At four weeks (d0), a superficial reference point was incised into the dorsal horn wall by carving a small indentation (estimated depth ≤0.5 mm) with a hoof knife, 0.5 cm below the coronary band (periople). This indentation was then coloured with Indian ink. After 15 days, the reference point was again coloured with Indian ink. At nine weeks (d36) the displacement above and below this reference point was measured using a digital calliper to determine horn growth (distance between periople and reference point at 9 weeks minus 0.5 cm), wear (length of dorsal border minus 0.5 cm at 4 weeks minus length of dorsal border form reference point to the claw bearing surface at 9 weeks, viz. apex of toe), and net horn growth (horn growth minus wear).

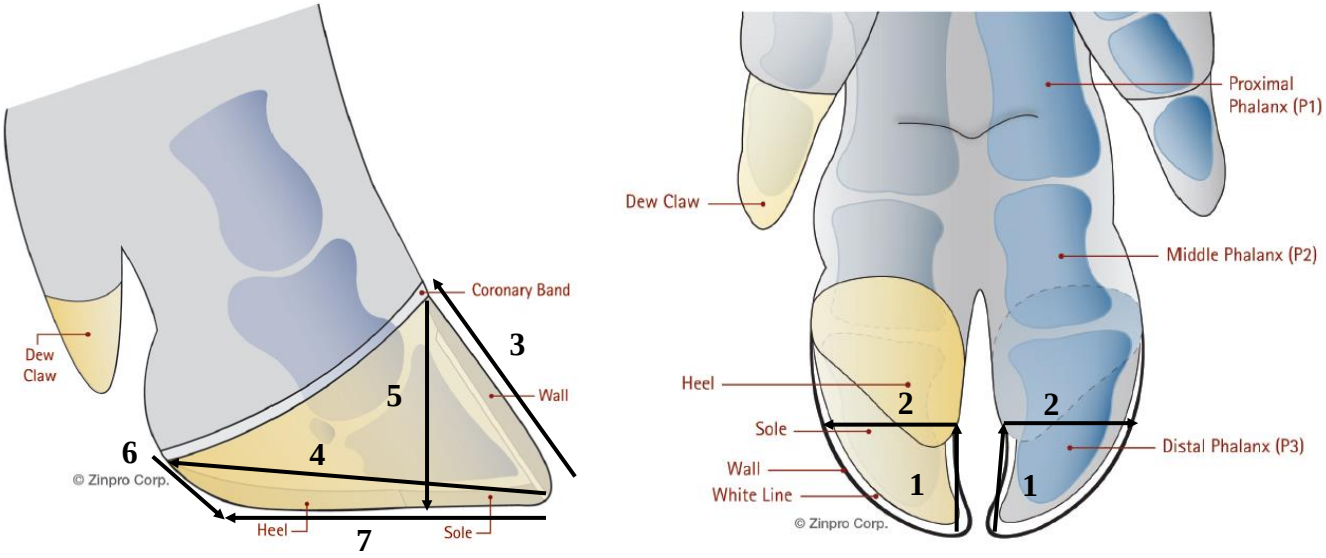

Figure 6.1. Representation of the claw dimension (mm) measurements in pigs. Claw dimension measurements were determined at front and hind claws, at the lateral and medial claw digits. (Reprinted with permission of © ZINPRO corporation, Eden Prairie, MN, USA, www.zinpro.com). 1= sole (base) length, determined at the inside of the digit from the heel/sole junction until the apex of the toe; 2= claw width, determined as the distance from the heel/sole junction at the inside of the digit (digital calliper is kept parallel to measurement 1) to the outside wall of the digit; 3= length of dorsal border, from coronary band (periople) at the dorsal claw area to apex of the toe; 4= diagonal claw length, a straight line from periople at the outer abaxial side of the claw to the apex of the toe; 5= toe height, perpendicular distance from dorsal periople to claw weight bearing surface; 6= heel height, parallel distance to the claw from periople at the outer abaxial side of the claw to surface; 7= claw length, claw surface (endpoint measure 6) to apex of toe.

After blood sampling and claw dimension measurements, the sedated piglets were euthanased using 2.5 mL/piglet of T61® (200 mg Embutramide, 50 mg Mebenzoniumiodide, and 5 mg Tetracaïne hydrochloride; Intervet International B.V.). Claw samples were collected *post mortem*. Using a surgical knife, a claw horn wall (abaxial) sample from the periople to horn wall and heel horn sample closest to the heel horn/sole junction (both containing the epidermal and dermal layer) of each claw digit were taken and used for histological examination (Figures 6.2 and 6.3). The sampling site of the abaxial horn wall was chosen, because horn production, initiated at the periople, is an important process for an optimal claw quality and Zn may influence this horn production. The sampling site of the heel horn was chosen, because the claw is more susceptible to develop claw lesions at the junction between hard and soft claw areas (Anil *et al.,* 2007; Ossent, 2010).

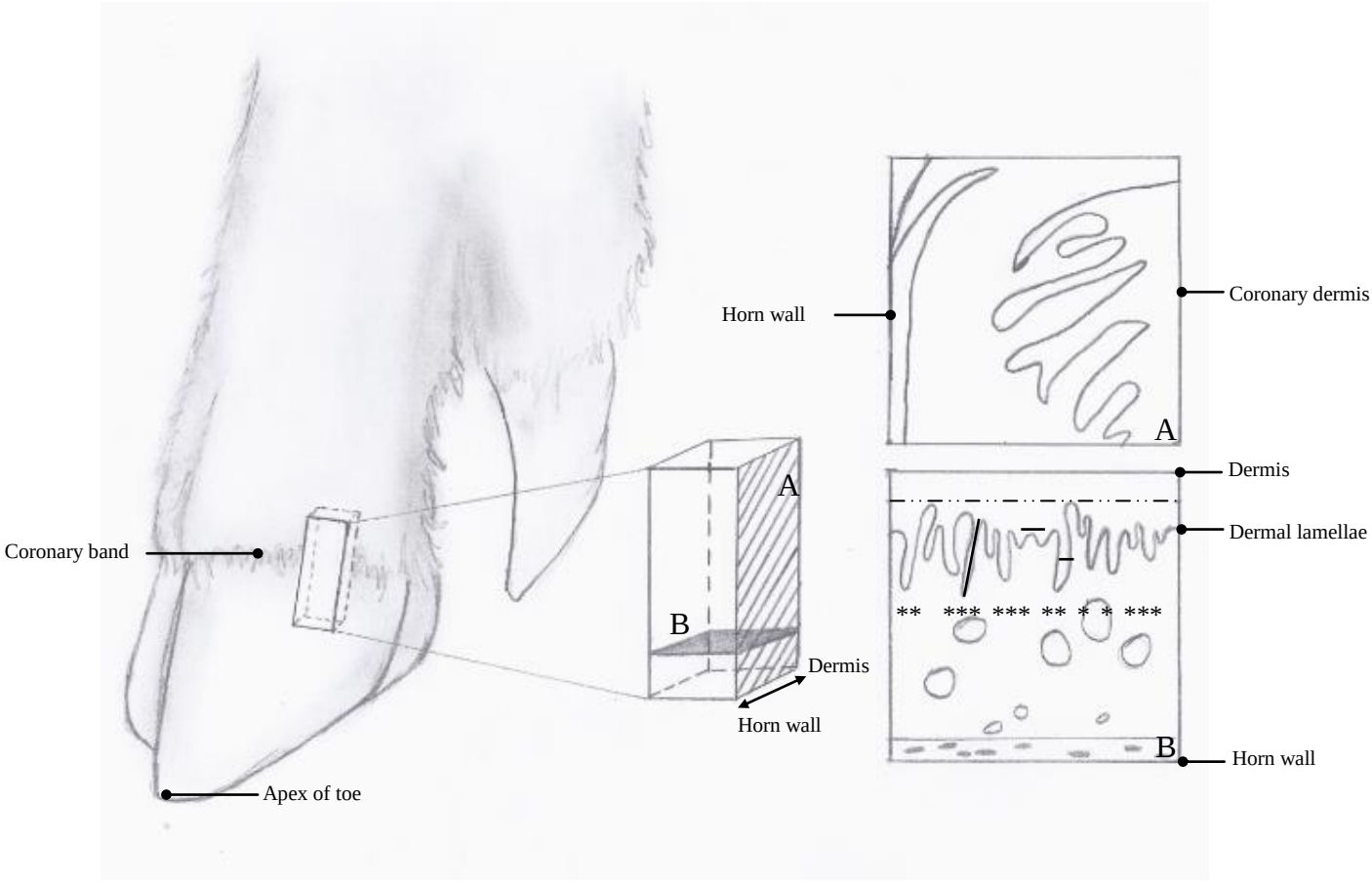

Figure 6.2. Horn wall sample collection and measurement of the transverse histological claw characteristics in pigs. Horn wall sample collection from coronary band (periople) to cornified horn wall representing the sagittal side (A) and transverse side (B). Samples were collected preferably from non-damaged or unaffected tissues. A, sagittal horn wall section representing the coronary region (coronary dermis and epidermis), not included for histological evaluation; B, transverse horn wall section including: number of dermal lamellae (*) (visible at their full width) corrected for section length (−··−) (standardised at 1000 µm); distance between dermal lamellae (distance between the axis lines at their base, µm), represented as straight line between the 7th and 8th lamellae; dermal lamellae width (dermal component halfway and perpendicular to the lamellae axis, µm), represented as straight line at the 9th lamellae; length of the longest dermal lamellae (measured from the top of the lamellae to the origin at the base, µm), represented as straight vertical line at the 4th lamellae.

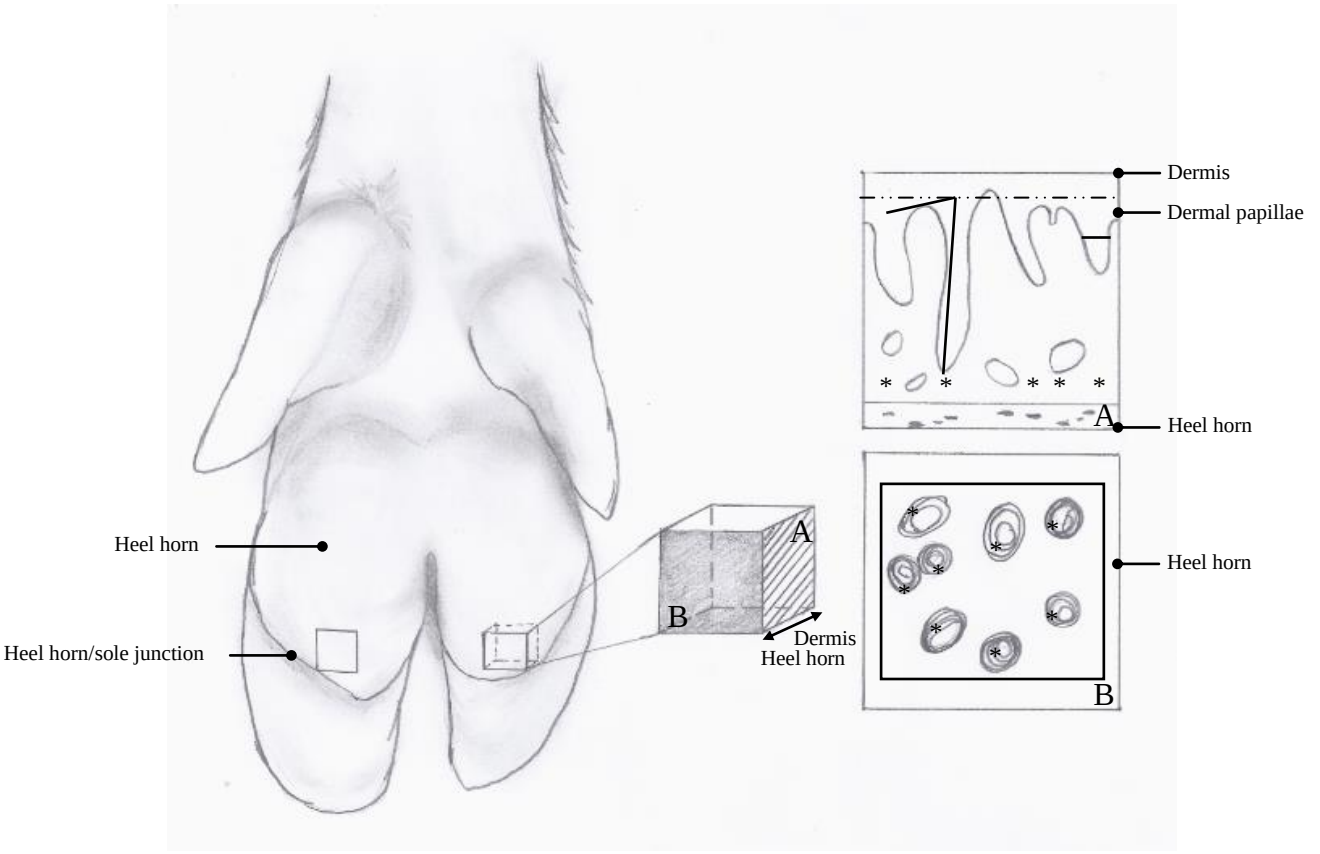

Figure 6.3. Heel horn sample collection and measurement of the sagittal and transverse histological claw characteristics in pigs. Heel horn sample collection closest to the heel horn/sole junction representing the sagittal side (A) and transverse side (B). Samples were collected preferably from non-damaged or unaffected tissues. A, sagittal heel horn section including: number of dermal papillae (*) (visible at their full width) corrected for section length (−··−) (standardised at 1000 µm); distance between dermal papillae (distance between the axis lines at their base, µm), represented as straight line between the 1st and 2nd papillae; dermal papillae width (dermal component halfway and perpendicular to the papillae axis, µm), represented as straight line at the 5th papillae; length of the longest dermal papillae (measured from the top of the lamellae to the origin at the base, µm), represented as straight vertical line at the 2nd papillae. B, transverse heel horn section including: density of heel horn tubules expressed as number of horn tubules (*) within a defined surface area (represented as square) for each section (1 mm^2). Horn tubules that were only partially visible from two of the four sides of the defined surface area were also included.

Preparation and analyses of blood samples

One millilitre of heparinised blood per piglet was used to determine the haematocrit percentage (centrifuged at 2749 *g* for 30 min at 20 ºC). The remainder of the heparinised sample was centrifuged (at 1500 *g* for 10 min at 4 ºC). The obtained plasma was divided among several 5 mL disposable polystyrene tubes and stored for 24 h at -20 ºC, then transferred to storage at -80 ºC until analysis for Zn and Cu concentrations.

Plasma samples were deproteinated (Randox ZN2607, Randox Laboratories Ltd., Crumlin, UK) by mixing them with an equal volume of trichloroacetic acid and centrifuged for 10 min at 10,000 *g*. The remaining supernatant was used within 2 h to determine plasma Zn and Cu concentrations. For plasma Zn, the deproteinated plasma was diluted five times with a colour reagent (Randox kit, ZN2341, Randox Laboratories Ltd., Crumlin, UK), and incubated for 5 min at 25 ºC. The absorbance was measured at a wavelength of 570 nm with a reference wavelength of 620 nm using a microplate reader (EZ reader 400, Biochrom Ltd., Cambridge, UK). The observed plasma Zn concentration was interpolated from the standard multipoint calibration curve. The inter- and intra-assay coefficients of variability were 2.19 and 3.61 %, respectively. The minimum and maximum recovery was 99.5 and 119.8 %, respectively (van Riet *et al.*, 2015). For plasma Cu, a reagent (Randox kit, CU2340, Randox Laboratories Ltd., Crumlin, UK) was added to the deproteinated plasma and this solution was incubated for 60 seconds at 37 ºC (Memmert Elanco incubation oven, Memmert GmbH, Schwabach, Germany). The absorbance was measured at a wavelength of 580 nm (Ultrospec IIE, LKB Biochrom Ltd., Cambridge, UK). A colour reagent (chromogen) was added, the solution mixed and after 5 min of incubation at 37 ºC the absorbance was measured at the same wavelength. The plasma Cu concentration was interpolated from the multiple standard calibration curve. The inter-assay coefficient of variability was 2.83 %. The minimum and maximum recovery was 96.4 and 103.7 %, respectively (van Riet *et al.*, 2015).

Preparation and analyses of claw samples

The horn wall and heel horn samples of left lateral and right medial claw digits were fixated in a 3.5 % buffered formaldehyde solution. The samples were stored for 24 h at room temperature and subsequently dehydrated in series of alcohol, cleared in xylene, and embedded in paraffin using an automated tissue processor (Microm STP 420D, Prosan N.V., Merelbeke, Belgium) and embedding station (Microm EC 350-1 and 350-2, Prosan N.V., Merelbeke, Belgium). Paraffin samples were hemi-sectioned and the resulting pieces were oriented and embedded in paraffin blocks so that either sagittal (perpendicular to the bearing surface) or transverse (parallel to the bearing surface) sections could be obtained.

A droplet of a 5 % potassium hydroxide (KOH) solution was applied, if necessary, immediately before sectioning on the surfaces of the paraffin blocks containing horn wall samples in order to soften the horn and allow for smoother cutting. Tissue sections of 8 µm thick were cut from all paraffin blocks using a microtome (Microm HM 360, Prosan N.V., Merelbeke, Belgium). The sections were harvested on uncoated slides and subsequently stained with haematoxylin (Hematoxylin, C.I. 75290, Merck KGaA, Darmstadt, Germany) and eosin (Eosine yellow, C.I. 45380, VWR International bvba/sprl, Leuven, Belgium) according to standard laboratory protocols.
The tissue sections were examined using a motorised microscope (Olympus BX 61, Olympus Belgium, Aartselaar, Belgium), which was linked to a digital camera (Olympus DP 50, Olympus Belgium, Aartselaar, Belgium). Standardised photographs of the transverse horn wall (20x magnification of the objective), and of both sagittal and transverse heel horn sections (10x magnification of the objective) were taken from five positions *(i.e.* yielding four replicates). Photographs of the transverse horn wall and sagittal heel horn sections were assessed once by one of the two observers randomly. One observer assessed all transverse heel horn sections. The number of dermal lamellae or papillae (visible at their full width) corrected for section length (standardised at 1000 µm) was determined on the transverse horn wall and sagittal heel horn sections, as well as the distance between the dermal lamellae/papillae (distance between the axis lines at their base), dermal lamellae/papillae width (dermal component halfway and perpendicular to the lamellae/papillae axis), and length of the longest dermal lamellae/papillae. The density of the heel horn tubules was determined on the transverse heel horn sections and expressed as the number of horn tubules within a defined surface area for each section (mm^2). The horn tubules that were only partially visible from two of the four sides of the defined surface area were also included (Figures 6.2 and 6.3).
Seventy-nine percent (79.2 %) of the transverse horn wall sections, 87.5 % of the transverse heel horn sections, and 63.5 % of the sagittal heel horn sections were included to calculate the above mentioned histological claw characteristics. The remaining sections were excluded due to broken samples, absence of the dermis layer, and sections containing living tissues of the heel horn or lamellae of the horn wall.

Statistical analysis

Blood biomarkers were analysed using a linear model with diet as fixed effect.
Claw conformation data were analysed using a linear mixed model with time of measurement (4 or 9 weeks), diet, digit (medial or lateral), claw (front and hind), and their interactions with time of measurement as fixed effects. Toe (left and right) was excluded from the final model if not

significant. In order to facilitate the interpretation of the model estimates, separate post-hoc contrasts at 4 and at 9 weeks of age were tested for diet, digit and claw.

For horn growth and wear, diet, digit, and claw were included as fixed effects. For the histological data, diet, digit, claw, and the interaction between digit and claw were included as fixed effects and a similar mixed Poisson model was used for the number of dermal lamellae/papillae. Interactions between digit and claw were excluded from the final model if not significant. A random effect for piglet was added to the models to correct for the repeated measurements at 4 and 9 weeks (d0 and 36) within piglet.

The analysed data (except the number of dermal lamellae/papillae) were considered to be sufficiently normally distributed, based on the graphical evaluation (histogram and QQ-plot) of the residuals. All analyses were performed using SAS 9.4 (SAS Institute Inc.).

The claw dimension "claw width" of the left front lateral claw digit of one piglet at 4 weeks was excluded from the analysis, because this observation was considered as an outlier compared to the same measurement of the other claws for this particular piglet. The claw horn size, claw volume, and sole area related to this specific measurement were also excluded. Furthermore, the diagonal claw length of right font lateral and medial digits at 4 weeks of one piglet, including the related claw horn size, were excluded for similar reasons.

Results

Performance

The bodyweight of the non-supplemented and Zn-supplemented piglets increased to nine weeks of age; 9.3± 0.3 and 9.2± 0.3 kg at 4 weeks, 13.1± 0.9 and 13.5± 0.9 kg at 6 weeks, and 27.7± 1.9 and 28.6± 2.1 kg at 9 weeks, respectively. The average daily feed intake over the entire period was 0.76± 0.03 kg. The average daily gain (ADG) was 0.51 kg ± 0.1 kg for the non-supplemented piglets and 0.54 kg ± 0.1 kg for the Zn-supplemented piglets. The clinical health of the piglets was not affected throughout the experiment.

Blood biomarkers

Haematocrit (%) was not different between non-supplemented (34.3± 0.6 %) and Zn-supplemented piglets (32.3± 1.3 %; P=0.164).

Plasma Zn concentration was lower in the non-supplemented piglets (58.1± 4.0 µg/dL) compared to the Zn-supplemented piglets (82.3± 6.1 µg/dL) (P=0.003), whereas plasma Cu concentration did not differ between the non-supplemented (156.4± 5.2 µg/dL) and Zn- supplemented piglets (158.5± 5.7 µg/dL) (P=0.794).

Table 6.3. Claw dimensions (mm) of Zn-supplemented (n= 12) and non-supplemented (n= 12) piglets during a 5-week experimental period.

Claw	Sole length		Claw width		Claw length				Toe height		Heel height		Claw length	
					Dorsal border		Diagonal							
	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn
							4 weeks							
Avg	9.3	9.5	11.1	11.1	18.9	18.8	24.8	24.1	15.9	15.6	6.6	7.5	20.1	18.9
Front	9.2	9.4	11.4	11.6	18.9	18.6	25.2	24.5	16.2	15.6	6.8	7.8	20.5	19.3
Hind	9.4	9.6	10.9	10.7	18.9	19.1	24.4	23.7	15.5	15.7	6.4	7.3	19.6	18.4
L	9.1	9.5	11.3	11.4	18.9	18.8	25.1	24.0	16.2	15.9	6.6	7.4	20.0	18.7
M	9.4	9.6	10.9	10.9	18.9	18.8	24.5	24.2	15.6	15.4	6.6	7.7	20.2	19.0
SEM	0.1		0.1		0.1		0.1		0.1		0.1		0.1	
P														
Diet	0.275		0.915		0.800		0.006		0.199		<0.001		0.001	
Claw	0.270		<0.001		0.273		0.001		0.105		0.045		0.010	
Digit	0.349		0.005		0.921		0.493		0.002		0.594		0.521	
							9 weeks							
Avg	15.2	15.2	15.7	15.4	29.7	29.3	37.0	36.3	21.8	21.3	11.6	11.2	28.3	28.2
Front	14.4	14.4	16.3	16.1	28.5	28.3	36.8	36.0	21.9	21.5	11.7	11.4	28.7	28.4
Hind	16.0	16.0	15.1	14.7	30.9	30.4	37.2	36.6	21.7	21.1	11.5	11.1	27.9	28.0
L	15.4	15.5	16.6	16.2	29.9	29.5	37.5	36.8	22.6	21.8	11.8	11.5	28.9	28.4
M	15.0	14.9	14.8	14.6	29.5	29.1	36.5	35.8	21.0	20.8	11.3	10.9	27.7	28.0
SEM	0.1		0.1		0.2		0.2		0.1		0.1		0.2	
P														
Diet	0.880		0.028		0.092		0.007		0.010		0.119		0.782	
Claw	<0.001		<0.001		<0.001		0.043		0.075		0.270		0.092	
Digit	0.020		<0.001		0.103		<0.001		<0.001		0.020		0.012	

Claw length, length of the dorsal border and diagonal claw length; +Zn, mean of Zn-supplemented piglets, Zn added as ZnO to the diet; -Zn, mean of non-supplemented piglets, Zn originating only from ingredients; Avg, average of all piglets per dietary treatment group; SEM, standard error per claw dimension at 4 and 9 weeks, including both non-supplemented and Zn-supplemented piglets; L, lateral claw digits; M, medial claw digits; Diet, differences between non-supplemented and Zn-supplemented diet; Claw, differences between front and hind claws; Digit, differences between lateral and medial digits.

Claw conformation

Claw dimensions

Initially at 4 weeks of age (d0), the non-supplemented piglets had a lower diagonal claw length (-0.7 mm; P=0.006), higher heel height (+0.9 mm; P<0.001), and shorter claw length (-1.2 mm; P=0.001) compared to the Zn-supplemented piglets. The diagonal claw length continued to be lower for the non-supplemented piglets at 9 weeks (-0.7 mm; P=0.007), whereas other effects disappeared (Table 6.3).

At 9 weeks of age (d36), the non-supplemented piglets had a narrower claw width (-0.3 mm; P=0.028), and a lower toe height (-0.5 mm; P=0.010) compared to the Zn-supplemented piglets.

The length of the dorsal border tended to be lower for the non-supplemented piglets (-0.4 mm; P=0.092). The sole (base) length was not different between the non-supplemented and Zn-supplemented piglets (P>0.100) (Table 6.3).

Claw morphology calculations

Initially at 4 weeks of age (d0), the non-supplemented piglets had a smaller sole area (claw length × claw width) (-13.3 mm^2; P=0.045), and smaller toe:heel ratio (-0.4 mm; P<0.001) compared to the Zn-supplemented piglets. These differences were no longer observed at 9 weeks of age (P=0.088 and P=0. 723, respectively) (Table 6.4).

At 9 weeks of age (d36), the non-supplemented piglets had a smaller claw volume (sole area × heel height) (-297 mm^3; P=0.003), and a smaller claw horn size (claw width × diagonal claw length) (-22.7 mm; P<0.001) compared to the Zn-supplemented piglets. No differences were found for distal toe angle (P=0.325) between the non-supplemented and Zn-supplemented piglets (Table 6.4).

Claw horn growth and wear

At 9 weeks of age (d36), horn growth and wear were lower for the non-supplemented piglets compared to the Zn-supplemented piglets (-1.7 mm; P=0.044 and -2.0 mm; P<0.001, respectively), whereas net horn growth (horn growth minus wear) did not differ between the two dietary treatment groups (P=0.406) (Table 6.5).

Table 6.4. Calculated claw dimensions* of Zn-supplemented (n= 12) and non-supplemented (n= 12) piglets during a 5-week experimental period.

Claw	Distal toe angle		Sole area		Claw volume		Claw horn size		Toe: heel ratio	
	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn
			4 weeks							
Avg	58.0	57.0	223	210	1474	1582	277	268	2.5	2.1
Front	60.4	58.1	234	223	1587	1734	290	284	2.5	2.1
Hind	55.6	55.9	213	197	1362	1433	265	253	2.6	2.2
L	59.8	58.5	226	213	1494	1582	285	273	2.6	2.2
M	56.3	55.5	220	207	1455	1581	270	263	2.5	2.1
SEM	0.5		2.1		27.5		2.2		0	
P										
Diet	0.228		0.045		0.272		0.152		<0.001	
Claw	<0.001		<0.001		0.008		<0.001		0.159	
Digit	<0.001		0.382		0.849		0.051		0.089	
			9 weeks							
Avg	47.9	47.1	446	435	5155	4858	583	560	1.9	2.0
Front	50.9	50.0	469	456	5450	5138	600	578	1.9	1.9
Hind	45.0	44.2	424	414	4859	4578	565	542	2.0	2.0
L	49.8	48.2	481	462	5675	5277	624	597	1.9	2.0
M	46.0	45.9	411	408	4634	4439	541	522	1.9	2.0
SEM	0.4		5.0		76.1		5.1		0	
P										
Diet	0.325		0.088		0.003		<0.001		0.723	
Claw	<0.001		<0.001		<0.001		<0.001		0.669	
Digit	0.001		<0.001		<0.001		<0.001		0.896	

+Zn, mean of Zn-supplemented piglets, Zn added as ZnO to the diet; -Zn, mean of non-supplemented piglets, Zn originating only from ingredients; Avg, average of all piglets per dietary treatment group; L, lateral claw digits; M, medial claw digits; Diet, differences between non-supplemented and Zn-supplemented diet; Claw, differences between front and hind claws; Digit, differences between lateral and medial digits.

* Distal toe angle (º), calculated based on sine of the length of the dorsal border and toe height; Sole area (mm^2), claw length × claw width; claw volume (mm^3), sole area × heel height; claw horn size (mm^2), claw width × diagonal claw length; and toe: heel ratio (mm), toe height : heel height.

Table 6.5. Horn growth and wear (mm) of Zn-supplemented (n= 12) and non-supplemented (n= 12) piglets during a 5-week experimental period.

Measurement*	+Zn		-Zn		*P*
	Mean	SE	Mean	SE	
Horn growth	18.2	0.4	16.8	0.3	0.044
Wear	7.3	0.3	5.3	0.3	<0.001
Net horn growth	10.9	0.3	11.4	0.2	0.406

+Zn, mean of Zn-supplemented piglets, Zn added as ZnO to the diet; -Zn, mean of non-supplemented piglets, Zn originating only from ingredients.
* Horn growth, distance between periople and reference point at 9 weeks – reference point (0.5 cm) at 4 weeks; Wear, (dorsal border length at 4 weeks – 0.5 cm reference point) – distance between reference point and claw bearing surface (apex of toe) at 9 weeks; Net horn growth, horn growth minus wear.

Histological claw characteristics

To assess differences between the two observers, both observers conducted histological measurements of 10 sagittal heel horn sections using an average of five photographs for each section. A paired t-test was used to analyse these differences. No differences between observers were found for the sample length (P=0.508), dermal number (P=0.705), longest length (P=0.305), and distance between papillae (P=0.484). Differences were found for the papillae width (P=0.002, CI 8.3, 2.7 µm). However, the mean difference (5.5 µm) between observers was considered irrelevant and had no biological importance. This was confirmed by the inter-observer reliability, using Pearson correlation, showing correlation coefficients of 0.63 for sample length, 0.94 for dermal number, 0.98 for longest length, 0.79 for distance between papillae, and 0.94 for papillae width. Transverse horn wall (n= 53) and sagittal heel horn (n= 55) sections with four replicates per section were used to compare the number of dermal lamellae/papillae present in the sections between the two observers. This number was chosen because the distance between lamellae/papillae and width of the lamellae/papillae are interrelated with the number of lamellae/papillae observed. The number of identified dermal lamellae/papillae in both horn wall and heel horn sections differed between the two observers (P<0.001, CI: 0.26, 0.09). The mean difference (0.17) between observers was considered irrelevant and had no biological importance. Based on these results, the statistical model did not correct for differences between observers.

Transverse horn wall

At 9 weeks of age (d36), no significant differences were found between non-supplemented and Zn-supplemented piglets for the number of dermal lamellae per 1000 µm (P=0.201), distance between

lamellae (*P*=0.123), width of the lamellae (*P*=0.210), or length of the longest lamellae (*P*=0.242) (Table 6.6, Figure 6.4).

Table 6.6. Histological claw characteristics* of formaldehyde fixated transverse horn wall sections (n= 76) of Zn-supplemented (n= 12) and non-supplemented (n= 12) piglets during a 5-week experimental period.

Item	Lamellae		Distance		Width		Length	
	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn
Avg	14.0	12.8	70.6	81.7	19.6	23.3	162.7	179.8
Front	14.8	13.2	65.0	80.9	16.8	21.0	151.6	172.5
Hind	13.2	12.3	76.2	82.5	22.5	25.7	173.8	187.0
L	14.2	13.8	68.6	73.3	18.4	20.6	166.9	173.8
M	13.8	11.9	72.6	89.3	20.9	25.8	158.5	185.1
SEM	0.4		2.9		1.1		6.6	
P								
Diet	0.201		0.123		0.210		0.242	
Claw	0.101		0.289		0.022		0.179	
Digit	0.099		0.063		0.053		0.885	
d*C†	NS		NS		NS		NS	

+Zn, mean of Zn-supplemented piglets, Zn added as ZnO to the diet; -Zn, mean of non-supplemented piglets, Zn originating only from ingredients; Avg, average of all piglets per dietary treatment group; L, lateral, interrelated to left front and hind claws; M, medial, interrelated to right front and hind legs; Diet, differences between non-supplemented and Zn-supplemented diet; Claw, differences between front and hind claws; Digit, differences between lateral and medial digits; d, digits (lateral or medial); C, claw (front or hind).

* Lamellae, number of dermal lamellae per 1000 µm, visible at their full width; Distance, distance between the axis lines of the lamellae at their base (µm); Width, width of the dermal component halfway and perpendicular to the dermal lamellae (µm); Length, length of the longest lamellae measured from the top of the lamellae to the origin at the base (µm).

† Interaction between digit (lateral or medial) and claw (front or hind) were excluded from the model if not significantly different.

Sagittal heel horn

At 9 weeks of age (d36), no significant differences were found between non-supplemented and Zn-supplemented piglets for the number of dermal papillae per 1000 µm (*P*=0.827), distance between papillae (*P*=0.339), width of the papillae (*P*=0.417), or length of the longest papilla (*P*=0.329) (Table 6.7, Figure 6.4).

Transverse heel horn

At 9 weeks of age (d36), the density of the heel horn tubules, expressed as the number of horn tubules within a defined surface area for each sample, was not different between non-supplemented and Zn-supplemented piglets (*P*=0.974) (Table 6.7, Figure 6.4).

Table 6.7. Histological claw characteristics* of formaldehyde fixated sagittal (n= 61) and transverse heel horn sections (n= 84) of Zn-supplemented (n= 12) and non-supplemented (n= 12) piglets during a 5-week experimental period.

Item	Papillae		Distance		Width		Length		Horn tubules	
	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn
Avg	4.3	4.2	220.4	232.8	83.1	79.1	552.6	600.6	19.7	20.2
Front	4.1	4.2	222.8	237.3	80.3	72.2	577.1	692.3	19.4	19.2
Hind	4.4	4.2	218.7	229.2	85.0	84.6	535.9	526.1	19.9	21.0
L	3.8	4.0	241.7	239.1	82.3	79.9	675.6	605.6	18.6	18.8
M	4.7	4.5	199.1	226.1	83.9	78.2	429.7	595.3	20.7	21.5
SEM	0.1		6.3		2.3		23.3		0.7	
P										
Diet	0.827		0.339		0.417		0.329		0.974	
Claw	0.512		0.643		0.072		0.033		0.313	
Digit	0.008		0.053		0.932		0.004		0.027	
d*C†	0.006		0.014		NS		NS		NS	

+Zn, mean of Zn-supplemented piglets, Zn added as ZnO to the diet; -Zn, mean of non-supplemented piglets, Zn originating only from ingredients; Avg, average of all piglets per dietary treatment group; L, lateral, interrelated to left front and hind claws; M, medial, interrelated to right front and hind legs; Diet, differences between non-supplemented and Zn-supplemented diet; Claw, differences between front and hind claws; Digit, differences between lateral and medial digits; d, digits (lateral or medial); C, claw (front or hind).

* Papillae, number of dermal papillae per 1000 µm, visible at their full width; Distance, distance between the axis lines of the papillae at their base (µm); Width, width of the dermal component halfway and perpendicular to the dermal papillae (µm); Length, length of the longest papillae measured from the top of the lamellae to the origin at the base (µm); Horn tubules, heel horn tubules density expressed as number of horn tubules within a defined surface area for each section (1 mm^2). Horn tubules that were only partially visible from two of the four sides of the defined surface area were also included.

† Interaction between digit (lateral or medial) and claw (front or hind) were excluded from the model if not significantly different.

Results for the effects between lateral and medial claw digits and between front and hind claws are presented as supplemental information.

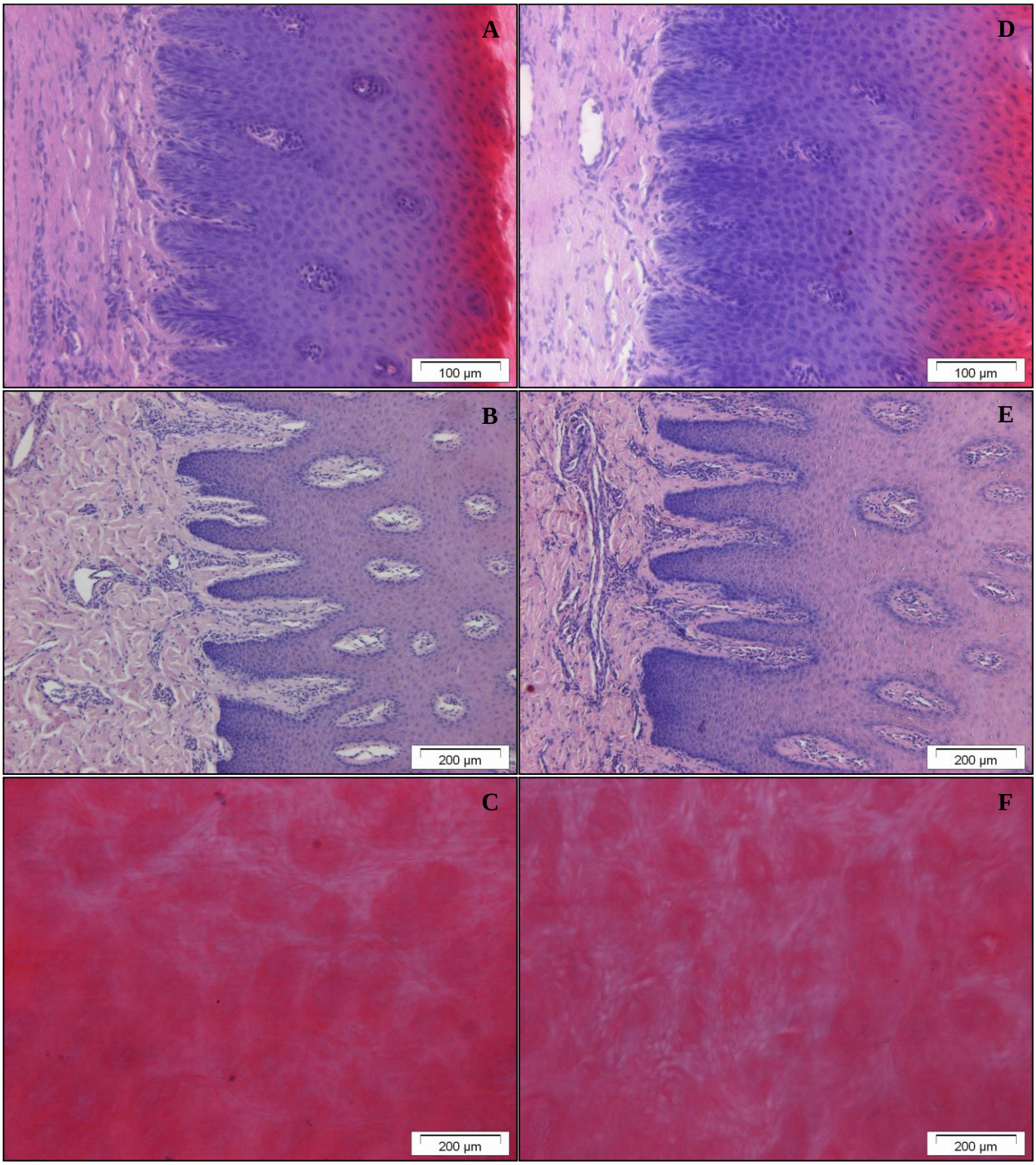

Figure 6.4. Transverse and sagittal horn wall and heel horn sections of non-supplemented (n= 12) and Zn-supplemented piglets (n= 12)*. A, B and C, claw sections of non-supplemented piglets; D, E and F, claw sections of Zn-supplemented piglets; A and D, transverse horn wall sections (20x magnification of the objective); B and E, sagittal heel horn sections (10x magnification of the objective); C and F, transverse heel horn sections (10x magnification of the objective). * No significant differences between the two dietary treatment groups.

Discussion

The results showed that marginal dietary Zn concentrations affected claw conformation but not histological claw characteristics in piglets within 5 weeks post-weaning. Additionally, differences between lateral and medial claw digits and between front and hind claws were found, irrespective of dietary Zn concentration. Unfortunately, initial claw conformation differed between the two dietary treatment groups. This makes it hard to unambiguously differentiate between differences originating from the initial conformation or from the dietary treatment. Still, some of the initial differences were unaffected, while other differences disappeared and new differences occurred over time. Causal factors that initiated these differences at the start of the study remains unclear, because piglets were randomly allocated to the treatment groups. Potentially, observer bias may have (partly) interfered with these differences. Furthermore, it cannot be excluded that pen could have been a confounding factor, but since housing conditions were similar, this seems very unlikely and this potential bias was considered as negligible in our statistical analysis. It would be interesting to repeat the study to confirm the present result, including a higher number of piglets. Lastly, the present study was performed under experimental conditions simulating conventional settings. The addition of colistin to the diet to prevent diarrhoea is expected to have a positive influence on the intestinal integrity and consequently nutrient absorption, including Zn (Torrallardona *et al.,* 2003; Li *et al.,* 2008). Although, there are no indication that colistin influences Zn metabolism, it cannot be excluded that responses would differ in diets without colistin. However, we opted to use colistin to prevent post-weaning diarrhea as a major confounding factor for the study.

Compared to previous studies in cattle and sows (Kessler *et al.,* 2003; Enjalbert *et al.,* 2006; Anil, 2011), the present study included a wider range of claw quality measurements, except for mechanical properties. The exhaustive measurements contributed to a more thorough insight into the function of Zn during horn production, because claw quality is largely determined by the quality of horn production (*i.e.* adequate resistance to environmental challenges). Nevertheless, the fact that not all parameters reacted to dietary Zn concentrations emphasises the importance of selecting enough adequate parameters to ensure a reliable overall conclusion. These findings are in accordance with some other studies in cattle that also reveal different effects among measurements (Moore *et al.,* 1989; Stern *et al.,* 1998; Kessler *et al.,* 2003). Furthermore, existing literature is equivocal: some studies report that Zn supplementation affected claw quality in both deficient and non-deficient diets (*e.g.* cattle: Moore *et al.,* 1989; Enjalbert *et al.,* 2006; Siciliano-Jones *et al.,* 2008; sows: Anil, 2011), whereas others found no effect of Zn form or concentration (*e.g.* cattle: Griffiths *et al.,* 2007; Lethbridge, 2009; sows: Bradley, 2010), or even different effects in different

claw areas (*e.g.* cattle: Randy *et al.,* 1985). These results indicate that the conditions on how Zn can improve claw quality and for which claw areas Zn is important are not identified, despite its known function in keratinisation (Mülling *et al.,* 1999; Tomlinson *et al.,* 2004; Lethbridge, 2009).

The non-supplemented piglets were provided with a dietary Zn concentration below requirements (Van paemel *et al.,* 2010; Bikker and Jongbloed, 2014; EFSA, 2014), resulting in a decreased plasma Zn concentration compared to Zn-supplemented piglets. This lower plasma Zn concentration indicates that either less Zn is circulating in the body or that it is maintained in high priority tissues (not claws) to ensure Zn homeostasis (King, 1990; King *et al.,* 2000; Hess *et al.,* 2007; Lowe *et al.,* 2009). Therefore, less Zn may be available to maintain claw quality, which may be reflected in changed claw dimensions and lower horn growth and less wear. Other studies in cattle support this hypothesis: hoof growth was lower, hoof walls thinner, and connections and horn weaker during Zn deficiency (Patterson *et al.,* 2003; Bindari *et al.,* 2013).

The quality of keratinisation depends on the diffuse nutrient supply, and a perturbed nutrient supply may result in impaired horn production (Tomlinson *et al.,* 2004; Muelling, 2009). However, this could not be confirmed in the present study: histological claw characteristics did not differ between the non-supplemented and the Zn-supplemented piglets. We expected to see histological changes before other changes occurred, because the morphology of a claw is as important as its mechanical properties (Vincent, 1992, Winkler, 2005). In a study in fattening bulls, histological examinations (visualising horn tubules) were also not different between the control group (no Zn supplementation) and groups supplemented with Zn in either organic or inorganic form (Kessler *et al.,* 2003). In another study in beef cattle, an enlargement of the center of the tubules in the coronary horn was found in response to Zn supplementation in organic form (Stern *et al.,* 1998, Winkler, 2005). Three potential reasons for this absence of observed histological changes are that: 1) changes are only present in the structures that are affected in the presence of claw lesions (Geyer, 1979, Geyer and Troxler, 1988); 2) the effect of Zn is different depending on the specific claw areas; and 3) the Zn reserves of the piglets were high enough at the start of the experiment to allow changes in histological claw characteristics within a 5-week period.

The study duration is thus an important factor to consider (Griffiths *et al.,* 2007; Lethbridge, 2009). Some studies suggest that lack of effect of Zn on claw quality may be related to the study's duration being shorter than 12 months. The horn capsule, a component of the claw, is known to be produced in 12 to 30 months in cattle (Hedges *et al.,* 2001; Toni *et al.,* 2007; Lethbridge, 2009). However, we

observed macroscopic differences within 5 weeks, and these piglets had presumably started the study with an appropriate plasma Zn concentration, due to the active transfer of Zn from the sow (van Riet *et al.*, 2015; Matte *et al.*, 2014). A possible explanation, supported by the high horn growth and wear rates that we found, could be that young production animals grow faster and probably have a higher turnover rate of the claw horn compared to more mature animals such as those reported by Vermunt and Greenough (1995) and Greyer (1979). This would explain the differences we observed within 5 weeks. Despite our efforts to include a wide range of measurements, the contrast between the changes in claw conformation and the unchanged histological claw characteristics requires further investigation.

Conclusion

Claw conformation but not histological claw characteristics were affected by marginal dietary Zn concentration in piglets during a 5-week experimental period post-weaning. Albeit, the selected claw quality measurements differed in their response to dietary Zn concentration, the marginal dietary Zn concentration (non-supplemented, below Zn requirements) used in the present study may be insufficient to maintain claw quality in piglets. However, initial differences in claw conformation may have been (partly) interfered with the results observed at the end of the study. Further research is warranted to confirm the present results, including a higher number of piglets.

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Supplemental information

Observed differences between lateral and medial claw digits and front and hind claws in weaned piglets.

Claw conformation

Claw dimensions

Initially at 4 weeks of age (d0), lateral digits had a wider claw width (+0.4 mm; P=0.005), and higher toe height (+0.6 mm; P=0.002) compared to the medial digits. These differences were still observed at 9 weeks of age (Table 6.3).

At 9 weeks of age (d36), the lateral digits had longer sole (base) lengths (+0.5 mm; P=0.020), longer diagonal claw lengths (+1.0 mm; P<0.001), higher toe heights (+0.7 mm; P=0.007), higher heel heights (+0.6 mm; P=0.020), and longer claw lengths (+0.9 mm; P=0.012) compared to medial claw digits. The length of the dorsal border was not different between lateral and medial claw digits (P=0.103) (Table 6.3).

Initially at 4 weeks of age (d0), hind claws had a smaller claw width (-0.7 mm; P<0.001), shorter diagonal claw length (-0.8 mm; P=0.001), lower heel height (-0.5 mm; P=0.045), and shorter claw length (-0.9 mm; P=0.010) compared to the front claws. Differences between front and hind claws continued for claw width and diagonal claw length at 9 weeks (Table 6.3).

At 9 weeks of age (d36), the hind claws had longer sole (base) lengths (+1.6 mm; P<0.001) and longer dorsal border lengths (+2.2 mm; P<0.001) compared to the front claws. The toe height tended to be lower for the hind claws compared to the front claws (-0.3 mm; P=0.075) (Table 6.3).

Claw morphology calculations

Initially at 4 weeks of age (d0), the lateral digits had a greater distal toe angle (+3.2°; P<0.001) compared to the medial digits. The claw horn size tended to be different at 4 weeks (P=0.051), and differed at 9 weeks (P<0.001): lateral digits had a higher claw horn size (+78.4 mm^2) compared to the medial digits (Table 6.4).

At 9 weeks of age (d36), the lateral digits had a greater sole area (+61.6 mm^2; P<0.001) and greater claw volume (+940 mm^3; P<0.001) than the medial digits. The toe:heel ratio was not different between lateral and medial claw digits (P>0.050) (Table 6.4).

Initially at 4 weeks of age (d0), the hind claws had a smaller distal toe angle (-3.5°; P<0.001), smaller sole area (-23.6 mm^2; P<0.001), smaller claw volume (-263 mm^3; P=0.008), and smaller claw horn size (-27.9 mm^2; P<0.001) compared to the front claws. These differences were still

observed at 9 weeks of age. The toe:heel ratio (toe height : heel height) was not different between front and hind claws (P>0.050) (Table 6.4).

Claw horn growth and wear

At 9 weeks of age (d36), the lateral digits had higher rates of horn growth (+1.3 mm; P<0.001) and wear (+1.0 mm; P=0.005) and the hind claws tended to have higher rate of horn growth (+0.6 mm; P=0.091) and lower wear rate (-1.4 mm; P<0.001) compared to the front claws. The net horn growth was only greater for the hind claws compared to the front claws (+2.0 mm; P<0.001).

Histological claw characteristics

Transverse horn wall. At 9 weeks of age (d36), the lateral digits tended to have a higher number of lamellae per 1000 μm (+1.2; P=0.099), tended to have a shorter distance between lamellae (-9.9 μm: P=0.063) and tended to have a shorter width of lamellae (-3.8 μm: P=0.053) compared to the medial claw digits (Table 6.6). The width of lamellae was higher for the hind claws compared to the front claws (4.5 μm: P=0.022) (Table 6.6).

Sagittal heel horn. At 9 weeks of age (d36), an interaction was found between claw digit (lateral and medial) and claw (front and hind) for the number of dermal papillae per 1000 μm: lateral hind claws had a lower number of papillae per 1000 μm (-1.2; P=0.006), and the lateral hind claws had a higher distance between papillae (+61.2 μm; P=0.014) (Table 6.7). Length of the longest papillae was longer for the lateral digits (+129.1 μm; P=0.004). Length of the longest papillae was shorter for the hind claws (-94.7 μm; P=0.033) and the width of the papillae tended to be wider for the hind claws (+8.4 μm; P=0.072) compared to the front claws (Table 6.7).

Transverse heel horn. At 9 weeks of age (d36), the heel horn tubule density, expressed as number of horn tubules within a defined surface area for each sample (1 mm2), was lower for the lateral digits (-2.6; P=0.027) compared to the medial claw digits (Table 6.7). No differences in the transverse heel horn were found between front and hind claws (P=0.313) (Table 6.7).

Chapter 6b

Zinc supplementation and claw quality in sows

Manuscript: Long-term impact of zinc supplementation on zinc status and claw quality in sows group housed on different floor types.

In preparation

Abstract

Malnutrition and floor type have been reported as predisposing factors for claw lesion development in sows. The objective of the present study was to evaluate the impact of Zn supplementation on (reproductive) performance, Zn status and claw quality in sows group housed on different floor types during gestation (d28-d108). Six groups of sows were alternately divided into group housing on rubber top-layer floors or on concrete floors for three reproductive cycles. Within each group, sows were randomly allocated to a diet varying in Zn concentration (0, 50 or 100 mg added Zn/kg diet). Claw quality measurements were performed at d50 and d140 of every cycle. After weaning of the third cycle, sows more than 12 months included in the experiment were slaughtered, and both front claws and liver were collected for analyses of mineral concentration and morphological claw characteristics. No interaction effects between dietary Zn concentration and floor type on any of the outcome variables were observed ($P>0.050$). Dietary Zn concentration did not influence serum metallothionein (MT) concentrations ($P=0.771$) and Zn concentrations in blood plasma ($P=0.125$), liver ($P=0.297$), bone ($P=0.177$), and horn wall ($P=0.864$) as Zn status biomarkers. The 100 mg Zn/kg supplemented sows had better scores for heel horn erosion compared with the non-supplemented sows at d50 ($P=0.013$), but had a lower performance ($P<0.001$) and reproductive performance (BW of weaned piglets, $P=0.009$) and the distance between dermal papillae of the sagittal heel horn was higher ($P=0.003$). Heel height was higher for the 50 mg Zn/kg supplemented sows ($P=0.011$) and toe:heel ratio was higher for the non-supplemented sows ($P=0.018$). No effects were found for horn growth and wear, net horn growth and abaxial horn wall strength. Independent of dietary Zn supplementation, claw scores were better for some lesion types for sows housed on rubber floors. Claw conformation measurements differed between sows housed on rubber and concrete floors. Horn growth and wear were lower for sows housed on rubber floors at d50 ($P=0.001$ for both variables) and histological characteristics of the sagittal heel horn differed between floor types. Net horn growth and horn wall strength did not differ between floor types. These results indicate that dietary Zn concentration had a minor influence on claw quality in sows, whereas floor type had a more systematic impact on multiple claw quality measurements and may be an important factor in the prevention of claw lesions in group-housed sows. The lack of impact of Zn supplementation may (partly) be attributed to the high dietary Zn concentration of the lactation diet.

Introduction

Claw lesions are a common multifactorial disorder in sows, with malnutrition and floor type besides others as predisposing factors (Heinonen *et al.,* 2013; Pluym *et al.,* 2013a). Claw lesions partly define claw quality as claw quality is evaluated by visual scoring for claw shape, claw shape dimensions, claw lesion scoring, and measurement of structural, physical and biochemical properties of the claw horn (Politiek *et al.,* 1986; Vermunt and Greenough, 1995). In sows, claw quality is mainly determined by claw lesion scoring. Other measurements, including claw conformation, horn growth and wear and histological and mechanical claw characteristics are very rarely evaluated. Claw quality depends on the internally formed characteristics of the claw, including an optimal horn production influenced by the diffuse nutrient supply from the dermis to the avascular epidermis (Vermunt and Greenough, 1995; Tomlinson *et al.,* 2004; Muelling, 2009). If the nutrient supply is insufficient, nutrient diffusion to the avascular epidermis is disturbed and horn production affected, thereby increasing the susceptibility of the claw to damage from the environment (Tomlinson *et al.,* 2004; Muelling, 2009). The structural, regulatory and catalytic functions of Zn are related to the horn production (Tomlinson *et al.,* 2004; van Riet *et al.,* 2013). Dietary Zn intake may change the level of Zn status biomarkers, such as plasma Zn and body tissue Zn concentrations (Jongbloed *et al.,* 2010) and these in turn may influence claw quality. The responses of serum MT are rather unknown. However, results from previous studies in mainly cattle are inconclusive showing no effect or an improvement in claw lesion and lameness scores at varying dietary Zn concentrations (Enjalbert *et al.,* 2006; Griffiths *et al.,* 2007; Lethbridge, 2009). In weaned piglets, claw quality and plasma Zn concentrations were affected by dietary Zn level (Chapter 6a).

While dietary Zn concentration may affect claw quality internally, floor type also affects the development of claw lesions (Pluym *et al.,* 2013a). Floor type affects claw quality externally by increasing the occurrence of claw lesions when sows are housed on fully or partly slatted concrete floors, whereas straw bedding seem to lower the presence of claw lesions (Lethbridge, 2009; Pluym *et al.,* 2013a). The effect of a rubber top-layer is less well understood, except an increased risk for some type of lesions in one study (Calderón-Díaz, *et al.,* 2013).

Therefore, we hypothesised that dietary Zn concentration would influence Zn status and claw quality in sows, whereas floor type would influence mainly claw quality. The objective of this longitudinal study over three reproductive cycles was to evaluate the effect of dietary Zn concentration on Zn status biomarkers and claw quality measurements in sows housed on two different floor types during gestation. For the present study, multiple Zn status biomarkers and a

wide range of claw quality measurements from both front and hind claws during gestation and lactation were included.

Materials and methods

This longitudinal two-factorial experiment was conducted according to the institutional and national guidelines for the care and use of animals and all experimental procedures involving these animals were approved by ILVO's ethical committee for animal experiments (approval no. 2013/196, May 7th, 2013).

Animals and management

Six groups of sows were replaced per group at three-week intervals by non-lame primiparous sows (total n= 131 gilts, RA-SE Genetics). These gilts were purchased per group of sows over time (22± 4 sows per group) by the Institute for Agricultural and Fisheries Research (ILVO, management system of 3 weeks) and quarantined for 4 to 6 weeks before their first insemination (233± 12d old at insemination). The experimental period started ten days before the first insemination and the six successive production groups (3 weeks interval between groups) were monitored during three reproductive cycles. Bodyweight, backfat thickness and body condition score (BCS) at the start of the study were 149± 21 (SD) kg, 15.5± 3.6 (SD) mm, and 3± 0.5 (SD) respectively. Sows were vaccinated at d55 of gestation against porcine reproductive and respiratory syndrome (Porcilis®), seven and four weeks before parturition to prevent neonatal diarrhoea in piglets (Neocolipor®,) and one week postpartum against parvovirus (Parvoruvax®). The sows were dewormed 17d before parturition. In case a sow was removed (n= 36) before the end of the experiment, a new gilt replaced her. After weaning of the third reproductive cycle (end of the study), sows that had participated for at least 12 months (min. parity 2, n= 95) in the experiment were slaughtered in a commercial slaughterhouse. Twelve months was the threshold reported in literature for which a lack of effect of dietary Zn may have been related to a too short study duration (Hedges *et al.*, 2001; Griffiths *et al.*, 2007; Lethbridge, 2009). Both front claws were provided with a tie wrap of different colours to distinguish between left and right front claw. The sows were transported to the slaughterhouse in the afternoon the day before slaughter. The next morning, sows were slaughtered to collect both front claws cut off at the carpal joint before the scalding vat to preserve the metacarpal bone and claw structures and to collect whole liver. After collecting the claw structures for examination, the remaining part of the front claws, including metacarpal bones, were frozen at -20 °C.

Housing facilities

Sows were group housed on straw per group for 4 to 6 weeks during the quarantine period. The quarantine unit consisted of two compartments, the compartments were naturally ventilated and temperature to the outdoor temperature. The sows had artificial light when personnel entered the units and daylight between 07.30 and 15.30h.

Sows were housed in individual gestation crates shortly before the first insemination (d-10) and from weaning (d-7) until 4 weeks after insemination (d28) in their successive reproductive cycles. The compartments were naturally ventilated and temperature adapted according to the outdoor temperature. The sows had artificial light when personnel entered the units and daylight between 07.30 and 15.30h, except at the first week after weaning (lights on between 08.00 and 20.00h).

During mid- and end of gestation (d28-d108), the sows were housed in static groups provided with an automated feeding system with individual sow recognition through an electronic transponder in the sow's ear. The ventilation was set at 75 m^3/h/sow and indoor temperature was set at 20 °C. The sows had artificial light when personnel entered the units and daylight between 07.30 and 15.30h. In each compartment, one light source was turned on during the night so that sows could find and enter the feeding system easily. During behavioural observations, (data not shown) light sources were turned on for 24 hours for 5d. The group housing facility consisted of four compartments (4.45 m x 18.7 m). The two diagonally opposite compartments had a similar floor type: 1) concrete slats and solid concrete lying areas or 2) concrete slats covered with a rubber top-layer (EasyFix®, Rubber Products Ltd.) and 50 % of the solid concrete floor area covered with rubber mats (Gummiwerk Kraiburg Elastik GmbH & Co. Kg.), further indicated as concrete or rubber floor type. Groups were alternately assigned to the concrete or rubber floor type during group housing for the entire experiment. Two rubber balls and a static and dynamic brush per compartment were provided as environmental enrichment.

The sows were housed individually in farrowing crates from one week before expected parturition until weaning (d108-d143). The ventilation in the units with each 10 farrowing crates was adapted to the temperature. The indoor temperature was set to 23 °C. The sows had artificial light when personnel entered the units and daylight between 07.30 and 15.30h.

Dietary treatment

All purchased primiparous sows (gilts) were fed a pre-experimental gestation diet during the quarantine period (4-6 weeks) formulated according to NRC recommendations (NRC, 2012) and commercial standards for gestating sows. This pre-experimental diet contained 895 g/kg DM, 127 g/kg crude protein, 302 g/kg NDF, 155 g/kg ADF, 24 g/kg ADL, 28 g/kg crude fat, 70 g/kg crude

ash, 129 g/kg starch, 66 g/kg sugar, 121 mg/kg Zn (originating from ingredients and 100 mg added Zn/kg, added as ZnO), and 4.7 g ileal digestible lysine per kg diet.

Throughout the experimental period, sows were fed a gestation and lactation diet formulated according to NRC recommendations (NRC, 2012) and commercial standards (Table 6.8 and 6.9), except for Zn. The gestation diet was provided seven days before the first insemination or after weaning of the preceding reproductive cycle (d-7) until 1 week before parturition (d108). The sows were fed twice daily from d-7 to the first 4 weeks of gestation (d28), in total 2.3 kg, whereas during mid- and end gestation (d28-d108), sows were fed 2.6 kg per day. The lactation diet was provided from 1 week before parturition until weaning. The feed allowance of the week before parturition was 3 kg per day provided in two equal portions. After parturition, 0.25 kg of feed per piglet was gradually supplemented in addition to 3 kg feed, also provided in two equal portions daily.

Throughout the experiment, all sows had ad libitum access to drinking water, except in the first 4 weeks of gestation in which water was automatically provided through nipple drinkers for 15 min every hour and for 45 min while feeding in order to reduce water spillage.

Within each static group of sows, sows were randomly allocated to one out of three dietary treatment groups with an equal number of sows per treatment group depending on the number of sows. The dietary treatments differed in Zn concentration: 1) Zn not supplemented, Zn originated from ingredients only, 2) 50 mg Zn/kg supplemented, and 3) 100 mg Zn/kg supplemented. Zinc was supplemented to the diet 50 % as inorganic Zn source and 50 % as organic Zn source. Inorganic Zn was added as ZnO (75 % Zn) (33.3 or 66.6 g ZnO per 1000 kg feed, INVE Belgium N.V., Baasrode, Belgium), and organic Zn as Availa®Zn containing Zn (10 %) in an amino acid complex: single amino acids from hydrolysed soy proteins (molar ratio 1:1, 250 or 500 g Availa®Zn per 1000 kg feed, Zinpro Corporation, Eden Prairie, MN, USA).

Feed samples of the gestation and lactation diets were collected from every batch and ground to pass a 1 mm sieve for NIRS evaluation and composed per dietary treatment group every 3 months for proximate analysis according to international standard methods accredited by ISO 17025 (2005). A homogenised sample was further ground to pass a 0.5 mm sieve and 3 out of 5 samples per dietary treatment were subjected to Zn and Cu analysis.

Table 6.8. Ingredient composition of the gestation and lactation diets.

Ingredients (g/kg fresh matter)	Gestation	Lactation
Wheat	180	213
Barley	180	100
Maize	152	250
Wheat middlings	150	23
Beet pulp	120	43
Soybean meal	89	166
Soybeans heated	-	12
Soybean oil	21	-
Alfalfa meal	47	94
Beet molasses	30	30
Premix 3 %*	30	-
Premix 2.75 %†	-	27.5
Lard	-	30
Limestone	-	9.4
L-Val	-	0.9
L-Thr	0.8	0.7
DL-Met	0.7	0.3
L-Lys HCL	0.1	0.5
L-Trp	-	0.1
Salt	0.05	-

* Premix 3 % without Zn included per kg diet for gestation diet: vitamin A (12499 IU), vitamin D3 (1995 IU), vitamin E (60 mg), vitamin K3 (2.0 mg), vitamin B1 (2.0 mg), vitamin B2 (5.0 mg), vitamin B5 (20 mg), vitamin B6 (4.0 mg), vitamin B12 (0.04 mg), vitamin B3 (35 mg), vitamin B11 (3.0 mg), biotin (0.4 mg), choline (282 mg), $C_5H_{14}CINO$ (325 mg), $FeSO_4*H_2O$ (Fe: 80 mg/kg), $CuSO_4*5H_2O$ (Cu: 10 mg/kg), MnO (Mn: 80 mg/kg), anhydrous $Ca(IO_3)_2$ (I: 2 mg/kg), Na_2O_3Se (Se: 0.4 mg/kg), Ca (5.3 g), P (0.3 g), Mg (0.2 g), Na (1.5 g), Cl (2.8 g), K (0.1 g), 3-phytase (1000 FYT), anhydrous trimethylglycine (275 mg), sepiolite (470 mg/kg), bentonite-montmorillonite (470 mg/kg), formic acid (5.2 mg/kg), propionic acid (49 mg/kg), citric acid (1.5 mg/kg), ethoxyquine (2.4 mg/kg), butylated hydroxy anisol (1.9 mg/kg).

† Premix 2.75 % without Zn included per kg diet for lactation diet: vitamin A (15015 IU), vitamin D3 (1501 IU), 25-hydroxycholecalciferol (0.01 mg), vitamin E (150 mg), vitamin C (100 mg), vitamin K3 (2.0 mg), vitamin B1 (2.0 mg), vitamin B2 (9.0 mg), vitamin B5 (25 mg), vitamin B6 (5.0 mg), vitamin B12 (0.03 mg), vitamin B3 (45 mg), vitamin B11 (5.3 mg), biotin (0.5 mg), choline (649 mg), $C_5H_{14}CINO$ (748 mg), $FeSO_4*H_2O$ (Fe: 150 mg/kg), $CuSO_4*5H_2O$ (Cu: 15 mg/kg), MnO (Mn: 50 mg/kg), anhydrous $Ca(IO_3)_2$ (I: 2 mg/kg), Na_2O_3Se (Se: 0.3 mg/kg), organic Se (0.1 mg/kg), Ca (3.6 g), P (1.6 g), Mg (0.6 g), Na (1.7 g), Cl (3.3 g), K (0.02 g), 6-phytase (1500 FYT), citric acid (2.5 mg/kg), ethoxyquine (6.7 mg/kg), butylated hydroxy anisol (1.1 mg/kg), propyl gallate (1.1 mg/kg).

Table 6.9. Analysed and calculated[*] nutrient composition of the gestation and lactation diets[†].

Chemical analysis (g/kg)	Gestation			Lactation		
	0	50	100	0	50	100
Dry matter	877.4	876.9	877.1	880.0	878.3	879.6
Crude ash	56.9	56.9	56.7	62.8	63.0	63.0
Crude protein	136.7	136.9	136.8	160.8	161.0	160.7
Crude fat	41.2	41.7	41.6	51.6	52.0	51.3
Crude fibre	64.5	65.0	66.3	58.1	61.1	58.7
Starch	277	270	268	313	304	314
Sugar	55.8	56.2	55.2	55.4	55.2	53.6
ADF	72.0	72.4	68.5	54.8	54.1	60.3
NDF	167	162	159	121	118	116
ADL	9.9	10.6	11.5	6.8	6.3	7.0
Ca	8.1	8.6	9.1	12.3	12.1	10.8
P	4.4	4.3	4.3	5.0	4.9	5.0
Cu, mg/kg[‡]	18.6	14.1	13.8	20.9	20.8	19.9
	(15-25)	(13-15)	(13-15)	(19-22)	(19-22)	(19-22)
Zn, mg/kg[‡]	46.6	81.9	124.4	128.9	184.3	229.0
	(45-49)	(77-91)	(119-132)	(116-137)	(167-209)	(206-256)
vP		2.5			3.4	
ID Lys		6.0			7.9	
ID Met		2.3			2.9	
ID Met + cys		4.0			4.2	
ID Thr		4.3			5.5	
ID Trp		1.2			1.6	
ID Arg		6.6			8.3	
ID Leu		7.6			10.1	
ID Ile		3.8			5.1	
ID His		2.7			3.3	
ID Val		4.5			6.6	
ID Phe		4.7			6.2	
NEv (MJ/kg)		9.0			9.4	

ADF, acid detergent fibre; NDF, neutral detergent fibre; ADL, acid detergent lignin; vP, digestible P; ID, ileal digestible; NEv, net energy for pigs.

[*] Chemical analyses of vP, ID amino acids and NEv are calculated according to the feed tables of the Centraal Veevoederbureau (CVB, the Netherlands), 2007.

[†] Dietary treatment is presented as 0, 50, or 100 mg Zn/kg supplemented to the diet during gestation and lactation.

[‡] Zn and Cu concentration are the average value of multiple feed samples analyses. Ranges for both mineral concentrations in the gestation and lactation diet over time are presented between brackets.

Measurements and samples

On all sows, performance characteristics (bodyweight, backfat thickness, body condition score, and reproductive performance), Zn status biomarkers (liver and bone mineral concentration) and claw quality measurements (claw lesion scores, claw conformation, and horn growth and wear) were determined. For some Zn status biomarkers (plasma Zn and Cu, serum MT concentration) and claw quality measurements (horn wall Zn concentration, and histological and mechanical claw characteristics), 36 sows (12 of each dietary treatment group and at least one of each static sow group) were selected. These sows were selected according to three criteria: 1) three reproductive cycles completed, 2) remained in their group of origin (*e.g.* the group the sows was allocated to at the start of the experiment), and 3) housed in their group during the entire gestation period (*e.g.* sow was not separated from the group during group housing) (Table 6.10).

Table 6.10. Schematic presentation of the observations throughout the reproductive cycle.

Category	Baseline*	Insemination			Gestation		Lactation†
	d-10	d0	d20	d28	d50	d108	d140/d143
Performances	BW BF BCS	BF BCS	BF BCS	BW BCS	BCS	BW BF BCS	BW BF BCS Reproduction
Zinc status biomarkers		Plasma Zn Plasma Cu Serum MT			Plasma Zn Plasma Cu Serum MT	Plasma Zn Plasma Cu Serum MT	Plasma Zn Plasma Cu Serum MT Liver mineral Bone mineral
Claw quality measurements	CL CC GW				CL CC GW		CL CC GW Horn wall Histology MCC

d, day of reproductive cycle; BW, bodyweight; BF, backfat thickness; BCS, body condition score; CL, claw lesion scoring; CC, claw conformation; GW, horn growth and wear; Histology, histological claw characteristics; MCC, mechanical claw characteristics.

* The baseline measurement (d-10) is performed only at the start of the study, ten days before the first insemination.

† Liver and bone mineral concentration, horn wall Zn and Cu concentration, and histological and mechanical claw characteristics were determined after slaughter at weaning of the third reproductive cycle.

Performance characteristics

Bodyweight (BW), backfat thickness, and body condition score (BCS) were determined at d-10 (baseline, start of the study) and then on d0 (insemination), d20 and/or d28, d108, and d140/d143

(weaning) of every reproductive cycle (Table 6.10). Backfat thickness was determined between the 3rd and 4th last rib, 7 cm from the left and right side of the vertebrae (P2 position). After P2 was lubricated, backfat measurements were determined alternately at the left and right sides (Renco Lean Meater-12 60566, Renco Corporation, Minneapolis, USA). If the difference between left and right was 2 mm or more, the measurements were up to three times repeated. The average thickness was used for further calculations. Body condition score was visually determined according to Evans (1978) using an ordinal scale including five categories from score 1 representing an emaciated condition to score 5 representing an overly fat condition. A BCS of three represents an ideal condition.

Reproduction performances (number of piglets born alive, average bodyweight (kg) of piglets born alive, number of stillborn piglets, number of weaned piglets, and average bodyweight (kg) of weaned piglets) were recorded, taken cross-fostering between dietary treatments and provision of creep feed (transitional feed) to the piglets from d10 postpartum into account.

Zinc status biomarkers

Zinc status biomarkers were determined after blood collection at insemination (d0), d50, d108, and d143 (weaning) every reproductive cycle and tissue collection after slaughter (Table 6.10). Blood samples (20 mL) were taken from all sows within a group before feeding in the morning (between 08.30 and 09.00h) after overnight fasting of at least 18 hours. Blood samples were collected from the jugular vein, using stainless steel needles and plastic syringes, and added to one heparin and one serum vacuum tube (Terumo Europe, Leuven, Belgium). One millilitre heparinised blood was used to determine haematocrit level (centrifuged 2749 g, 30 min, 20 ºC) as additional marker to monitor sows' health. The remainder was centrifuged (1500 g, 10 min, 4 ºC) and plasma divided over two 5-mL disposable polystyrene tubes. The tubes were stored for 24 hours at -20 ºC and then transferred to storage at -80 ºC until analysis of plasma Zn and Cu concentration. The vacuum serum tubes were centrifuged (1500 g, 10 min, 4 °C) after resting overnight at 4 °C to allow clotting. Serum samples were divided over 5-mL disposable polystyrene tubes, stored for 24 h at -20 °C and then at -80 °C until analysis of serum MT concentration. Plasma samples were deproteinated (Randox ZN2607, Randox Laboratories Ltd., Crumlin, UK) by mixing them with an equal volume of trichloroacetic acid and centrifuged for 10 min at 10,000 g. The remaining supernatant was used within 2 hours to determine plasma Zn or Cu concentrations. Plasma Zn concentration was determined spectrophotometrically (EZ reader 400, Biochrom Ltd., Cambridge, UK) using a commercial colorimetric diagnostic kit (Randox kit, ZN2341, Randox Laboratories Ltd., Crumlin, UK). The observed plasma Zn concentration was interpolated from the multipoint standard

calibration curve. The inter- and intra assay CV were 1.3 and 1.1 %, respectively. The minimum and maximum recovery was 99.5 and 119.8 %, respectively (van Riet *et al.*, 2015). Plasma Cu concentrations was determined spectrophotometrically (Ultrospec IIE, LKB Biochrom Ltd., Cambridge, United Kingdom) using a commercial colorimetric diagnostic kit (Randox kit, CU2340, Randox Laboratories Ltd., Crumlin, UK). The plasma Cu concentration was interpolated from the multipoint standard calibration curve. The inter- and intra assay CV was 1.8 and 1.1%, respectively. The minimum and maximum recovery was 96.4 and 103.7 %, respectively (van Riet *et al.,* 2015). Serum MT concentration was determined using competitive ELISA (Porcine Metallothionein (MT) Elisa kit, E07M0030, BlueGene Biotech CO., Shanghai, China). The serum MT concentration was interpolated from the multipoint standard calibration curve. Serum MT concentrations below the detection limit (0.1 ng/ml) were corrected using the equation: detection limit/$\sqrt{2}$. The inter- and intra assay CV was 3.3 and 2.0 %, respectively. The certificate of analysis reported a recovery between 94 and 103 %. For quality control, serum samples have been spiked with 5 ng/ml and 10 ng/ml MT. The recovery of spiked MT was 96.6 and 94.3 %, respectively.

Body tissues (liver and bone) were collected after slaughter for mineral analyses. Livers were cooled during transport. The left lateral and medial lobe (not including right lobe with gall bladder) were sliced and ground using a mincer with 4.5 mm sieve (Kenwood kMix stand mixer with food grinder). Then, a representative homogenised sample (189± 23 (SD) g) was collected in a Petri dish and stored at -20 °C until lyophilisation. After lyophilisation, liver samples were oven dried at 103 °C to a constant weight and Zn and Cu concentration analysed.

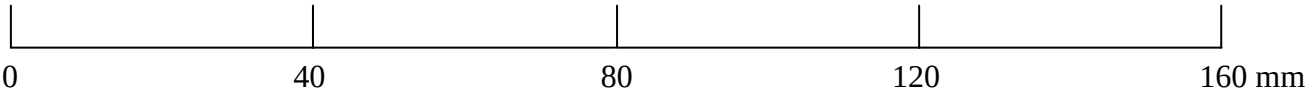
The right frozen front claws with tie wrap were placed in a beaker (500 ml) filled with warm water with the claws upwards to prevent charring of the metacarpal bones. The beaker was then placed in a warm water bath (75 °C) for 24 hours to soak. After 24 hours, the metacarpal bone 3 and 4 were collected and surrounding tissue removed. The metacarpi were weighed, oven dried for 16.45 hours at 65 °C (Drying oven Binder APT line series ED, Binder GMBH Tuttlingen, Germany), and weighed (Sartorius CP 324S, Göttingen, Germany) again. The length of the metacarpus 3 and 4 (standardised as the length of each bone at the interior side between metacarpi 3 and 4) was determined using a digital calliper. The metacarpi 3 and 4 were crushed in a vice using paper towels and stored at -20 °C until fat extraction. The crushed metacarpi 3 and 4 were defatted by extraction with petroleum ether (boiling point 40-60 °C, ISO 6492A), dried at 103 °C to a constant weight, and ashed at 825 °C to a constant weight according to the methodology described by Bikker *et al.* (2011, 2013). The ash content of the fat free dry matter was calculated based on the weighed bones before and after ashing. Metacarpi 3 and 4 were then ground according to a validated protocol not affecting Zn and Cu concentrations (data not shown) using a ball mill (Retsch PM100, Led Techno

NV, Heusden-Zolder, Belgium), a 125 ml stainless steel grinding jar, 7 stainless steel balls and 5 droplets of ethanol (p.a.) to obtain a homogenised sample. Grinding jar and balls were cleaned with alternately demineralised water (Type II), methanol (p.a) and demineralised water (Type II) to prevent contamination. Ground metacarpi 3 and 4 samples were composed (10 g each) to obtain one sample per sow and analysed for Ca, P, Zn and Cu content.

The left front claws were thawed for 24 hours and surrounding tissues removed using a surgical knife. The weight and dimensions (length, narrow and wide according to Figure 1 in Combs *et al.*, 1991) of metacarpus 3 and 4 were measured.

Claw quality measurements

Claw quality measurements, including claw lesion scoring, claw conformation measurements and horn growth and wear, were performed at d-10 (baseline) and then on d50 and d140 of every cycle (Table 6.10). For these measurements, sows were lifted in a sow chute (Zinpro Corporation, Eden Prairie, MN, USA) for maximum 15-20 min. The supporting beam of the sow chute was disinfected between sows at d140 to prevent transmission of pathogens. After cleaning and drying the claws, claw digits of front and hind claws were scored for 8 types of claw lesions using a tagged visual analogue scale (tVAS) (Figure 6.5) and 7 claw conformation measurements using a digital calliper (Mitutoyo Belgium N.V., Kruibeke, Belgium) (see Figure 6.1, Chapter 6a) following a methodology adapted from Calabotta *et al.* (1982) and Vermunt and Greenough (1995). These dimensions were subsequently used to calculate the distal toe angle (sine of the length of the dorsal border and toe height), sole area (claw length × claw width), claw volume (sole area × heel height), claw horn size (claw width × diagonal claw length), and toe: heel ratio (toe height : heel height) (Calabotta *et al.*, 1982; Vermunt and Greenough, 1995; Manske, 2002; Bradley, 2008; Van Amstel and Doherty, 2010).



Heel horn	Normal	Mild overgrowth and/or erosion	Multiple cracks with obvious overgrowth and erosion	Severe overgrowth and erosion with cracks
Heel/sole junction	Normal	Mild separation at the junction	Long extended separation at the junction	Long and deep extended separation at the junction
White line	Normal	Shallow and/or short separation along the white line	Long extended separation along the white line	Long and deep extended along the white line
Skin	Normal	Mild injury	Moderate/substantial injury	Inflammation periople
Horn wall	Normal	Haemorrhage and short shallow horizontal crack	Long shallow horizontal crack	Multiple and/or deep horizontal crack(s)
Horn wall	Normal	Short shallow vertical crack	Long shallow vertical crack	Multiple and/or deep vertical crack(s)
Claw length	Normal, ± 50 mm	Lateral and/or medial toe slightly longer	Lateral and/or medial toe considerably longer	Long toes that hamper locomotion
Dewclaw length	Normal, ± 20 mm	Dewclaws slightly longer	Dewclaws touch ground when sow is standing	Dewclaws cracked or partially/ completely torn off

Figure 6.5. Tagged visual analogue scale (tVAS) for claw lesion scoring in sows. Adapted and modified from the scorings guides of Wageningen University and FeetFirst (Zinpro Corp., Eden Prairie, MN). To score the claw area for claw lesions, a vertical bar was drawn on the line and the distance from 0 mm determined. The average distance of lateral and medial digits from front and hind claws per claw lesion type was used for further analyses. For skin lesion scoring, only skin lesions around the claw and dewclaw were included. Haemorrhages were included in scoring the horn wall for horizontal cracks. If haemorrhages are present, but no cracks, the score is 40 mm. The length of the dewclaw was determined by pushing the dewclaw against the claw and see if the dewclaw exceeds the heel height.

For horn growth and wear, a superficial reference point was incised into the dorsal horn wall of both claw digits of the left front and right hind claw by carving a small indentation with a hoof knife, 0.5 cm below the periople and coloured with Indian ink. At the subsequent evaluation (d50 or d140), the displacement above and below this reference point was measured using a digital calliper to

determine horn growth (distance between periople and reference point minus 0.5 cm), wear (length of dorsal border minus 0.5 cm of previous evaluation minus length of dorsal border from reference point to the claw bearing surface, viz. apex of toe), and net horn growth (horn growth minus wear). A new superficial reference point was incised into the dorsal horn wall and coloured with Indian ink.

Histological and mechanical horn characteristics, as claw quality measurements, were determined after slaughter. For histological examination, a claw horn wall (abaxial) sample including the periople and heel horn sample closest to the heel horn/sole junction (both containing the epidermal and dermal layer) of each claw digit of the front claws were collected using a surgical knife (Figure 6.6).

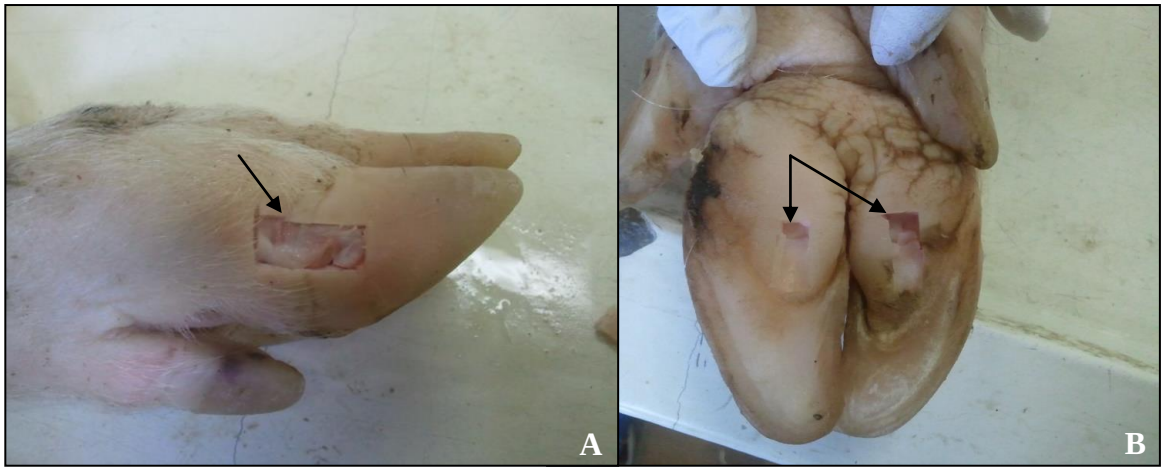


Figure 6.6. Location on the claw where abaxial horn wall samples including the periople (A) and heel horn samples closest to the heel horn/sole junction (B) of each claw digit of the front claws were collected to determine histological claw characteristics. Arrows represent location of the collected samples.

The horn wall and heel horn samples of left lateral and right medial claw digits were fixated in a 3.5 % buffered formaldehyde solution. The samples were stored for 24 hours at room temperature and subsequently dehydrated in series of alcohol, cleared in xylene, and embedded in paraffin using an automated tissue processor and embedding station (Microm, Prosan N.V., Merelbeke, Belgium). Paraffin samples were hemi-sectioned and the resulting pieces were oriented and embedded in paraffin blocks so that either sagittal (perpendicular to the bearing surface) or transverse (parallel to the bearing surface) sections could be obtained. The paraffin blocks were stored. Tissue sections of 8 µm thick were cut from the stored paraffin blocks using a microtome (Microm HM 360, Prosan N.V., Merelbeke, Belgium). A droplet of a 5 % KOH solution was applied, if necessary, immediately before sectioning on the surfaces of the paraffin blocks containing horn wall samples

in order to soften the horn and allow for smoother cutting. The sections were harvested on uncoated slides and subsequently stained with haematoxylin and eosin according to standard laboratory protocols. The tissue sections were examined using a motorised microscope (Olympus BX 61, Olympus Belgium, Aartselaar, Belgium), which was linked to a digital camera (Olympus DP 50, Olympus Belgium, Aartselaar, Belgium). Standardised photographs of the transverse horn wall and of both sagittal and transverse heel horn sections (10x magnifications) were taken from five positions (*i.e.* yielding four replicates). One of the two observers assessed once photographs of the transverse horn wall and sagittal heel horn sections alternately and one observer assessed all transverse heel horn sections. Ninety-three percent (93.4 %) of the transverse horn wall sections, 98.7 % of the transverse heel horn sections, and 71.1 % of the sagittal heel horn sections were included to calculate the histological claw characteristics, including number of dermal papillae/lamellae per 1000 µm, width, distance, and length of longest dermal papillae/lamellae, and horn tubules density (see Figures 6.2 and 6.3, Chapter 6a). The remaining sections were excluded due to broken samples and absence of the dermis layer. To assess differences between the two observers, both observers conducted histological measurements of 14 sagittal heel horn sections using an average of five photographs for each section. A paired t-test was used to analyse these differences. No differences between observers were found for the length of the longest dermal papillae (P=0.829) and width of papillae (P=0.129). Differences between observers were found for the sample length (P=0.031, CI 3.3-57.8 µm, mean difference 30.5 µm), number of dermal papillae (P=0.003, CI -0.9- -0.2, mean difference 0.6), number of dermal papillae per 1000 µm (P=0.003, CI -1.0- -0.2, mean difference -0.6), and distance between papillae (P=0.022, CI 9.1-100.3 µm, mean difference 54.7 µm). Based on these results, differences between observers were negligible and therefore removed from the final model. This was confirmed by the inter-observer reliability, using Pearson correlation, showing correlation coefficients of 0.95 for sample length, 0.79 for dermal number, 0.96 for longest length, 0.43 for distance between papillae, and 0.94 for papillae width.

For mechanical horn examination, an abaxial horn wall sample of the lateral and medial digits of the right front claw (mean length, width, thickness: 27x 17x 4.5 mm) was sawn using an oscillating saw and the underlining tissues removed with a surgical knife (Figure 6.7). The angle adjacent to the dorsal border and periople was marked. The horn wall samples were weighed and individually stored in vacuum (in order to prevent fluctuation in moisture content) at -20 °C until analyses for mechanical horn characteristics. Horn wall samples of the left front claw could not be used to test the mechanical horn characteristics due to the incised superficial reference point for horn growth

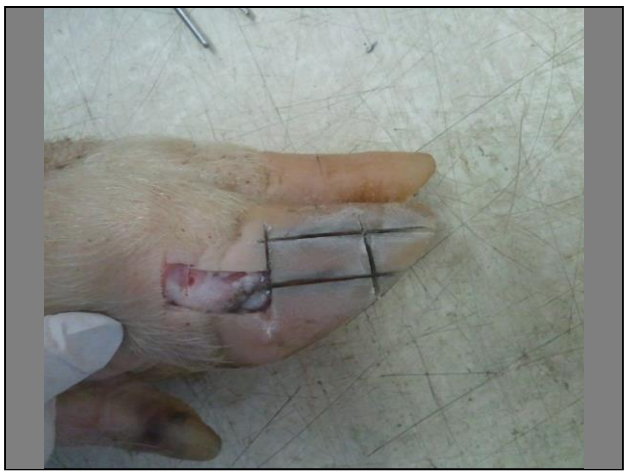


Figure 6.7. Location on the claw where the abaxial horn wall sample was collected using an oscillating saw to determine mechanical claw characteristics and mineral content. Square represents location of the collected samples.

and wear measurements. The lateral or medial abaxial horn wall samples (n= 36 sows, 1,7± 0.4 g) were defrosted for 24 hours at 4 °C and weighed (1.8± 0.4 g). The horn wall samples were cut into the required dimensions; 2 samples of 20 mm length and 6 mm width with a variable thickness using a mitre cutter with lever transmission (LOWE 3140/HÜ, Original LOWE, Gebr. Schröder GmbH, Kiel, Germany) and weighed. One sample had the marked angle adjacent to the dorsal border and periople and was tested in a similar order for each sample. The other lateral or medial sample of the same sow was tested the subsequent day in the opposite order than the previous day. The horn wall samples were tested with a three-point bending test (Texture Analyser (TA), Stable micro systems Ltd., Surrey, UK) according to the methodology described by Franck *et al.* (2006). The test characteristics (stress area and strain height) were adjusted for each sample, due to different sample thickness and weights. The span between the two supports was set to 15 mm and the sample was compressed over a distance of 5.5 mm (*e.g.* maximal deformation) using a force transducer (load cell, 30 kg) exerted in the middle of the span distance of 15 mm. Each sample was tested with a loading velocity of 1 mm/min and 15 mm/min to assess if the claw horn has visco-elastic properties. The time between the velocities ranged between 1 and 1.5 hour. A force-deformation curve was generated and converted (Exponent Software, Stable micro systems Ltd., Surrey, UK) to a stress-strain diagram. Then Young's modulus, yield stress, and maximal stress were determined (Franck *et al.,* 2006). Young's modulus is a measure for the rigidity and stiffness of the horn and represented as the slope of the linear phase of the initial line. Yield stress is the point on the stress-strain diagram in which the material starts to lose its mechanical function and material properties starts to change at further loading. Yield stress is represented as the point of the line where the line becomes nonlinear using a parallel straight line with the same slope as the initial line (strain equal to 1 %) where the intersect with the stress-strain diagram is defined as the yield stress (Franck *et al.,* 2006). Maximal stress is represented as the maximal load a sample can withstand (Franck *et al.,* 2006). The abaxial horn wall samples were stored in vacuum at -20 °C post-testing until further analysis of DM and Zn and Cu

concentration. Abaxial horn wall samples were dried at 103 °C to a constant weight and analysed for Zn and Cu content.

Chemical analysis

Feed, liver, bone, and horn wall samples were subjected to chemical analyses. The Zn, Cu and Ca concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Vista MPX, Varian Inc, Palo Alto, CA, USA) and bone P concentration was determined spectrophotometrically after matrix digestion. Feed samples (1 g) were ashed and digested with HNO_3 on a hot plate (150 °C) for at least 30 min and transferred to a 50 ml flask. Liver samples (0.25 g) were diluted with HNO_3/H_2O_2 for 12 hours and digested using microwave-assisted matrix digestion (MarsX, CEM, Matthews, NC, USA). Bone (1 g) was digested with 10 ml 6N HNO_3 in a flask and diluted until 50 ml, and horn wall samples (±0.8 g) were diluted in 10 ml 6N HNO_3 for 12 hours, heated on a hot plate (150 °C) for approximately 2 hours and transferred to a 50 ml flask.

Statistical analysis

Performance characteristics

Sows' performances (BW, BCS, backfat thickness) and reproductive performances were analysed using a linear mixed model with floor type, dietary Zn concentration, phase of the reproductive cycle, parity, and their interactions as fixed effects and reproductive cycle, sow and group as random effects. A similar Poisson mixed model was used for the reproductive performance characteristics related to the number of born piglets. Non-significant interactions were excluded from the final models.

Zinc status biomarkers

Blood biomarkers were analysed using a linear mixed model with floor type, dietary Zn concentration, phase of the reproductive cycle, parity, and their interactions as fixed effects and reproductive cycle, sow and group as random effects to correct for the repeated measurements. Non-significant interactions were excluded from the final models. Observations for serum MT were LOG transformed to obtain a normal distribution. For the mineral concentration in liver, bone and horn wall and bone characteristics a similar linear mixed model was used with floor type, dietary Zn concentration, parity, and their interactions as fixed effects.

Claw quality measurements

The mean claw lesions scores, claw conformation, and horn growth and wear were analysed using a linear mixed model with floor type, dietary Zn concentration, phase of the reproductive cycle and their interactions, parity, digit (lateral or medial), and claw (front and hind) as fixed effects and reproductive cycle, sow and group as random effects to correct for the repeated measurements. For the histological claw characteristic data, floor type, dietary Zn concentration, interaction between floor type and dietary Zn concentration, leg (left or right front), and digit (lateral or medial) were included as fixed effects and sow and group as random effects. For the mechanical claw characteristic data, floor type, dietary Zn concentration, interaction between floor type and dietary Zn concentration, and digit (lateral or medial) were included as fixed effects and sow and group as random effects per test velocity. Non-significant interactions were excluded from the final models. Difference between test velocities and between two samples of the same digit of the same sow were analysed using a paired sample t-test.

The analysed data (except reproductive performance characteristics related to the number of born piglets) were considered to be sufficiently normally distributed, based on the graphical evaluation (histogram and QQ-plot) of the residuals. In case of *post hoc* pairwise testing, p-values were corrected with the Tukey-Kramer adjustment for multiple comparisons. All analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

The interaction between dietary Zn concentration and floor type was not significant for any of the outcome variables ($P>0.050$). Therefore, effects of dietary Zn concentration and floor type, irrespective of the other main effect, are described. Effects of parity, claw (front and hind) and claw digits (lateral and medial) on claw quality measurements are presented as supplemental information.

Performance characteristics

Throughout three reproductive cycles, 36 sows (27.5 %, n= 9 non-supplemented sows, n= 16 and n= 11 Zn-supplemented sows from the 50 and 100 mg added Zn/kg treatment group, respectively) were removed from the experiment and 21 of them were replaced by a primiparous sow. Sows were removed for several reasons: 1) spontaneous death (n= 10), 2) euthanasia (rectal or uterus prolapse, severe locomotion disorders, n= 7), or 3) reproductive failure after multiple attempts (n= 19). Seventy sows remained in their group of origin, whereas 26 sows were transferred to another group allocated to the same treatment group and floor type. In total, 92 out of 95 sows were slaughtered.

Two sows died after their third parturition and front claws and liver from one sow were collected post mortem after euthanasia on the ILVO experimental farm directly after the rest of the group was loaded for transport.

For the sows' BW, an interaction between phase of reproductive cycle and dietary Zn concentration was found, irrespective of floor type, in which the BW of 100 mg Zn/kg supplemented sows was lower than the non-supplemented and 50 mg Zn/kg supplemented sows at d28, d108, and d143, but not at baseline ($P<0.001$, Figure 6.8). No differences in BW were found between the non-supplemented and 50 mg added Zn/kg diet dietary treatment groups. Body condition score and backfat thickness were also lower for the 100 mg Zn/kg diet supplemented sows (Table 6.11). Body condition score at d140 was lower ($P<0.001$) and backfat thickness at d108 was higher compared with other phases of the reproductive cycle ($P<0.001$). A parity effect was found for BW, BCS and backfat thickness ($P<0.001$), showing higher BW and lower BCS and backfat thickness in the third parity.

Dietary treatment was not associated with number of piglets born alive, average BW of piglets born alive, number of stillborn piglets, and number of weaned piglets (Table 6.11). Average BW of weaned piglets (kg) was influenced by dietary treatment; piglets from sows of 100 mg added Zn/kg diet showed a lower BW compared to non-supplemented and 50 mg added Zn/kg supplemented sows (Table 6.11). A parity effect was found for the number of stillborn piglets which was lower in the second parity ($P<0.001$) and for the average BW of piglets born alive which was lower in the first parity ($P<0.001$). The average BW of weaned piglets was lower in the third parity ($P<0.001$).

Haematocrit level (%) did not differ between dietary treatment groups (Table 6.11), but was lower at d108 and d143 compared with d0 and d50 of the reproductive cycle ($P<0.001$) and was lower at reproductive cycle 2 and 3 compared with reproductive cycle 1 ($P=0.003$ and $P<0.001$, respectively).

Independent of dietary Zn concentration, floor type did only have minor influences on reproductive performances (Bos *et al.*, unpublished data).

Table 6.11. Performance and reproductive performance characteristics of sows throughout three reproductive cycles for each dietary treatment group (n= 131)[*].

Characteristic	Dietary treatment[†]			SEM	*P*[‡]
	0	50	100		
BCS	3.2[a]	3.2[a]	2.9[b]	0.02	<0.001
Backfat (mm)	14.8[a]	14.4[a]	12.6[b]	0.20	<0.001
HCt (%)	37.1	36.5	36.8	0.47	0.654
Reproductive performance					
Piglets born alive (n)	13.5	14.3	13.5	0.31	0.130
Average bodyweight of piglets born alive (kg)	1.4	1.4	1.4	0.02	0.715
Stillborn piglets (n)	0.9	1.0	1.0	0.15	0.774
Weaned piglets (n)	11.0	11.4	10.9	0.28	0.439
Average bodyweight of weaned piglets (kg)	7.4[a]	7.5[a]	7.1[b]	0.10	0.009

BCS, body condition score; Backfat, Backfat thickness; HCt, Haematocrit level.

[*] Feed allowance was 2.3 kg at the beginning of gestation (d-7/d143-d27), 2.6 kg during mid- and end of gestation (d28-d108), 3.0 kg 1 week before parturition (d109-d115) and 3.0 kg +0.25 kg per piglet during lactation (d116-d143). Feed leftovers were negligible and not related to dietary treatment

[†] Dietary treatment is presented as 0, 50, or 100 mg Zn/kg supplemented to the diet during gestation and lactation.

[‡] There was no interaction between phase of the reproductive cycle and dietary treatment for BCS and backfat thickness, therefore the average BCS and backfat thickness are presented.

[a,b] Mean values within a row with unlike superscript letters were significantly different between dietary treatment groups ($P<0.050$).

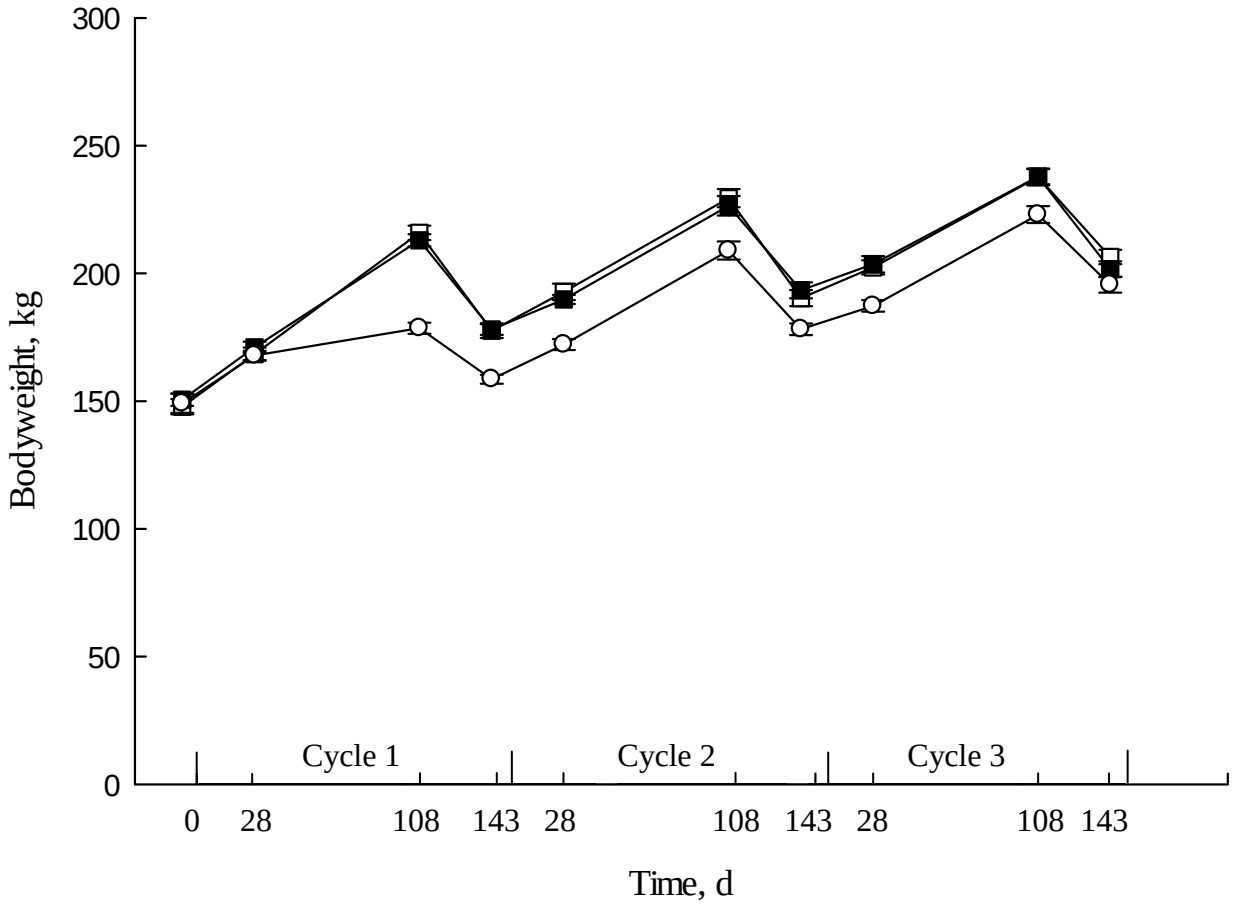


Figure 6.8. Fluctuations in bodyweight (kg) throughout three reproductive cycles in non-supplemented (–□–), 50 mg/kg (–■–) and 100 mg/kg (–○–) supplemented sows (n= 36, 12 of each dietary treatment group). Values presented are mean and their standard errors. Sows were group housed between d28 and d108 of gestation on different floor types. The BW of 100 mg Zn/kg supplemented sows was lower than the non-supplemented and 50 mg added Zn/kg supplemented sows at d28, d108, and d143 (*e.g.* interaction between phase of reproductive cycle and dietary Zn concentration, $P<0.001$). No differences were found between the non-supplemented and 50 mg added Zn/kg diet dietary treatment groups.

Zinc status biomarkers

Plasma Zn concentration did not differ between dietary treatment groups, irrespective of floor type ($P=0.125$). Plasma Zn concentration decreased from insemination (d0) to d50 of gestation ($P<0.001$), remained constant to d108 of gestation ($P=0.999$) and decreased further towards weaning (d143) ($P=0.003$). Plasma Zn concentration was lower in reproductive cycle 3 compared with reproductive cycle 1 and 2 ($P<0.001$ and $P<0.001$, respectively) (Figure 6.9).

Plasma Cu concentration did not differ between dietary treatment groups ($P=0.370$), but was lower at d143 compared with d0 and d50 ($P=0.005$ and $P=0.030$, respectively). Plasma Cu concentration

was lower in reproductive cycle 3 compared with reproductive cycle 1 and 2 (P=0.035 and P=0.008, respectively) (Figure 6.10).

Serum MT concentration did not differ between dietary treatment groups (P=0.771). It decreased from d0 towards d108 and increased towards d143 (P<0.001), but the MT concentration did not differ between d50 and d143 (P=0.285). The serum MT concentration was lower in reproductive cycle 2 compared with reproductive cycle 3 (P=0.025) (Figure 6.11).

Floor type did not affect plasma Zn and Cu concentrations (P=0.281 and P=0.694, respectively). Serum MT concentration was lower for sows housed on rubber floors (P=0.003).

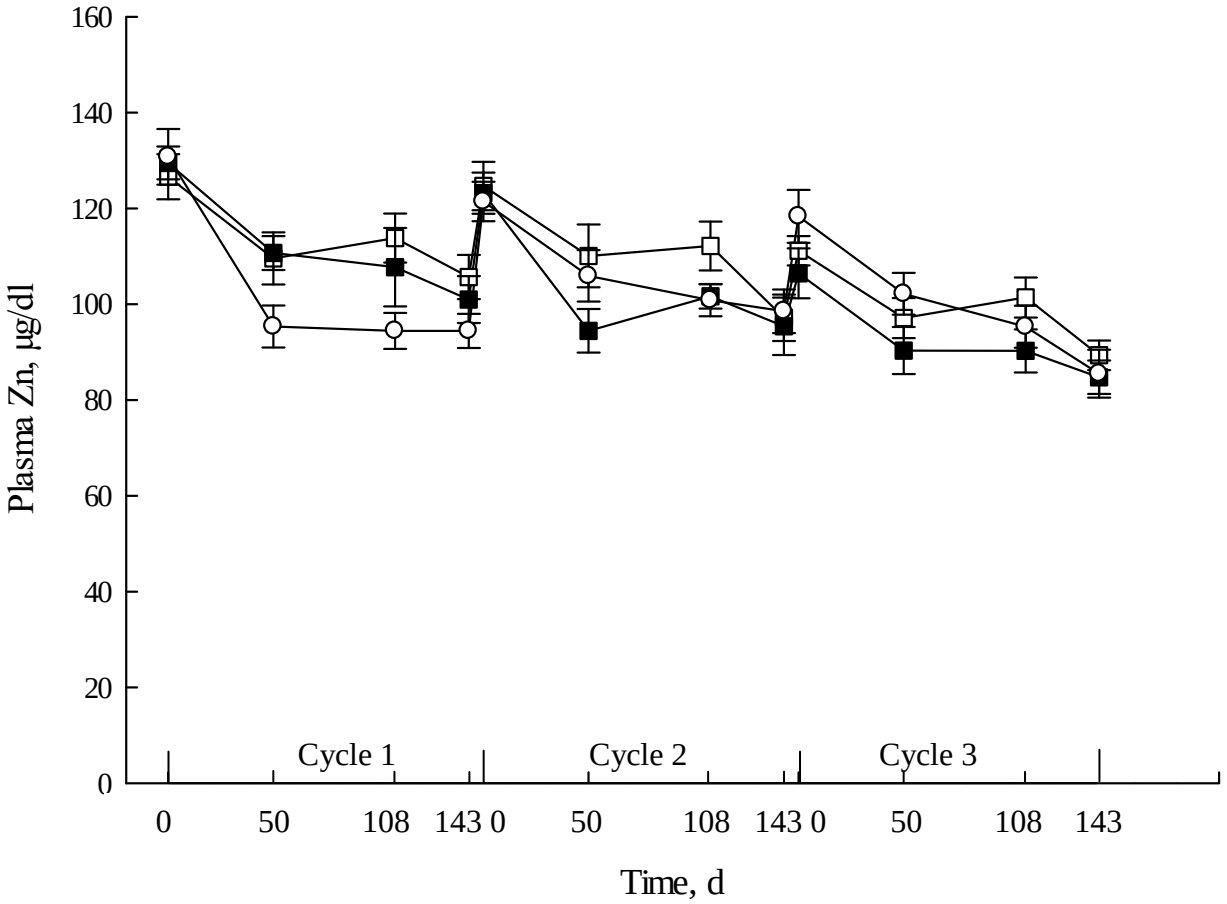


Figure 6.9. Fluctuations in plasma Zn concentrations (µg/dL) throughout three reproductive cycles in non-supplemented (–□–), 50 mg/kg (–■–) and 100 mg/kg (–○–) supplemented sows (n= 36, 12 of each dietary treatment group). Values presented are mean and their standard errors. Sows were group housed between d28 and d108 of gestation on different floor types. Plasma Zn concentration did not differ between dietary treatment groups (P=0.125) and between floor type (P=0.281). Plasma Zn concentration decreased from insemination (d0) to d50 of gestation (P<0.001), remained constant to d108 of gestation (P=0.999) and decreased further towards weaning (d143) (P=0.003). Plasma Zn concentration was lower in reproductive cycle 3 compared with reproductive cycle 1 and 2 (P<0.001 and P<0.001).

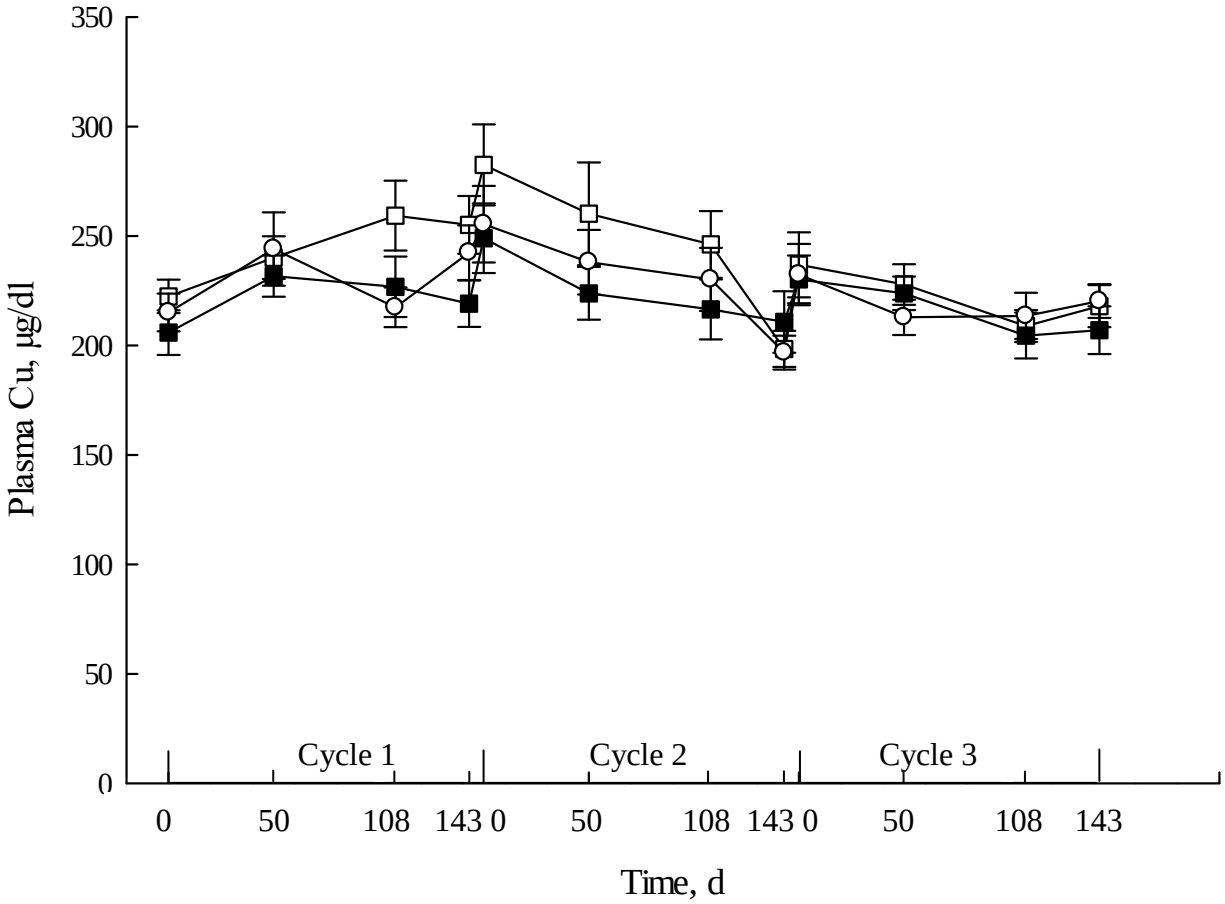


Figure 6.10. Fluctuations in plasma Cu concentrations (µg/dL) throughout three reproductive cycles in non-supplemented (–□–), 50 mg/kg (–■–) and 100 mg/kg (–○–) supplemented sows (n= 36, 12 of each dietary treatment group). Values presented are mean and their standard errors. Sows were group housed between d28 and d108 of gestation on different floor types. Plasma Cu concentration did not differ between dietary treatment groups (P=0. 370) and between floor type (P=0.694), but was lower at d143 compared with d0 and d50 (P=0.005 and P=0.030, respectively). Plasma Cu concentration was lower in reproductive cycle 3 compared with reproductive cycle 1 and 2 (P=0.035 and P=0.008, respectively).

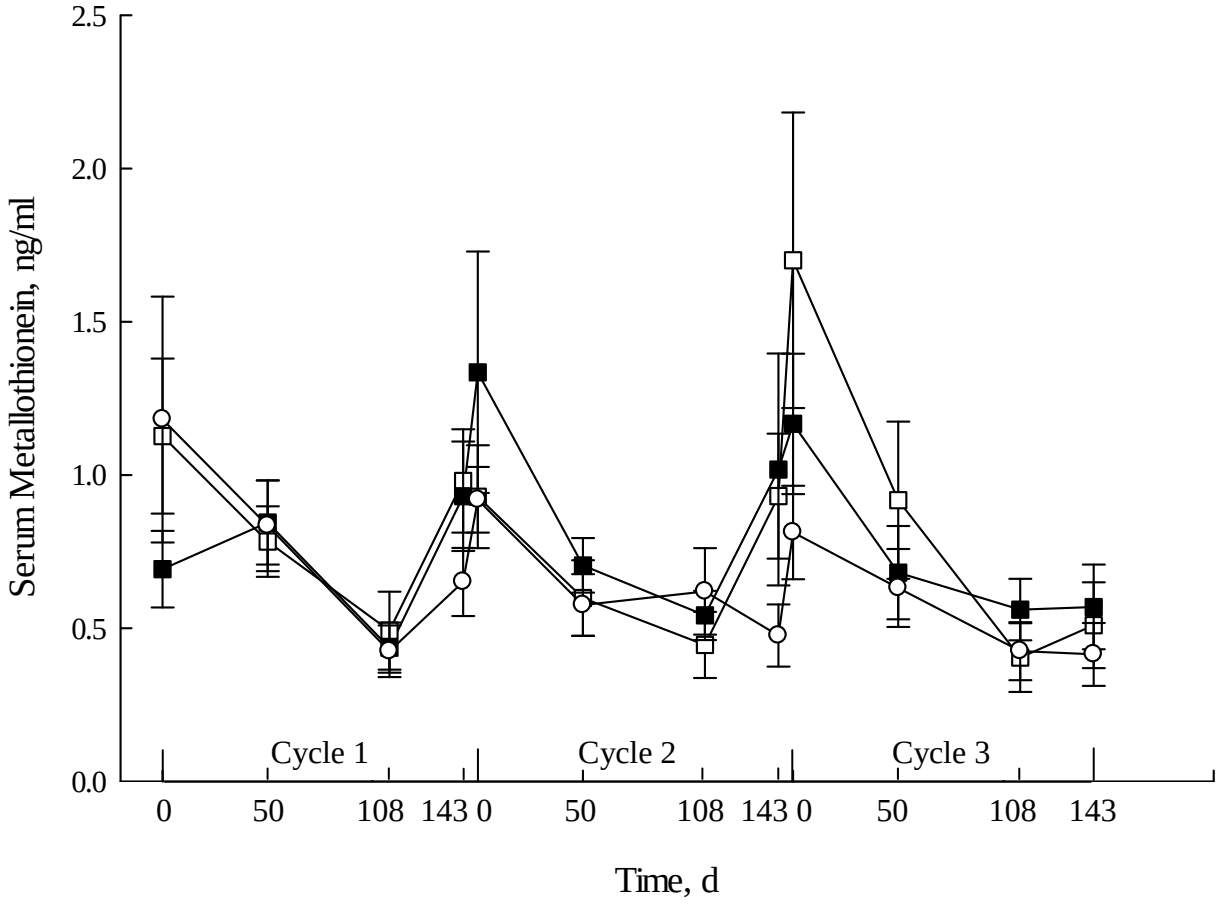


Figure 6.11. Fluctuations in serum MT concentrations (ng/mL) throughout three reproductive cycles in non-supplemented (–□–), 50 mg/kg (–■–) and 100 mg/kg (–○–) supplemented sows (n= 36, 12 of each dietary treatment group). Values presented are mean and their standard errors. Sows were group housed between d28 and d108 of gestation on different floor types. Serum MT concentration did not differ between dietary treatment groups (P=0.771), but was lower for sows housed on the rubber-top layer floor (P=0.003). Serum MT concentration decreased from d0 towards d108 and increased towards d143 (P<0.001), but the MT concentration did not differ between d50 and d143 (P=0.285). The serum MT concentration was lower in reproductive cycle 2 compared with reproductive cycle 3 (P=0.025).

Liver weight, liver Zn and Cu concentration, bone Zn, Cu, Ca and P concentrations, and horn wall Zn and Cu concentration did not differ between dietary treatment groups nor between floor type treatments (Table 6.12). Bone characteristics did also not differ between dietary or floor type treatment groups (Table 6.13).

Table 6.12. Effect of dietary zinc concentration on mineral concentration of body tissues from slaughtered sows group housed on different floor types between d28 and d108 of gestation (n= 94 sows for liver and bone, n= 36 sows for horn wall).

Body tissues	Dietary treatment*			Floor type		SEM	*P*†	
	0	50	100	Concrete	Rubber		Zn	Fl
Liver‡								
Total liver weight (g)	2841	2838	2686	2804	2758	54.9	0.425	0.590
Zn in fresh matter (mg/kg)	54.8	55.0	52.8	55.6	52.2	1.7	0.717	0.297
Total liver Zn (mg)	153.6	151.7	141.9	154.0	142.1	5.2	0.577	0.226
Cu in fresh matter (mg/kg)	34.7	37.9	33.8	31.5	40.2	3.6	0.926	0.221
Total liver Cu (mg)	98.2	107.3	92.6	86.6	114.3	10.7	0.890	0.210
Metacarpus 3 and 4								
Ash (g/kg FFDM)	627.6	620.5	620.2	620.6	626.0	0.2	0.277	0.252
Zn in ash (mg/kg)	60.6	52.1	59.4	55.2	61.2	2.2	0.280	0.193
Cu in ash (mg/kg)	9.3	8.7	9.1	9.3	9.0	0.3	0.487	0.799
Ca in ash (g/kg)	395.0	395.2	396.1	396.0	394.6	0.8	0.860	0.396
P in ash (g/kg)	180.3	182.2	180.0	180.5	181.0	0.5	0.149	0.636
Zn in FFDM (mg/kg)	38.2	32.4	36.9	34.4	38.4	1.4	0.262	0.177
Cu in FFDM (mg/kg)	5.8	5.4	5.7	5.8	5.7	0.2	0.404	0.899
Ca in FFDM (g/kg)	247.9	245.2	245.6	245.8	247.0	0.9	0.432	0.551
P in FFDM (g/kg)	113.1	113.1	111.7	112.0	113.3	0.5	0.384	0.240
Horn wall Zn								
Dry matter (g/kg)	765	767	775	764	774	3.6	0.456	0.393
Zn in DM (mg/kg)	128.0	122.2	120.4	123.0	124.0	2.3	0.432	0.864
Cu in DM (mg/kg)	3.9	4.0	3.8	4.1	3.7	0.2	0.943	0.423

Zn, zinc; F, floor type; FFDM, fat free dry matter.

* Dietary treatment is presented as 0, 50, or 100 mg Zn/kg supplemented to the diet during gestation and lactation.

† There were no interactions between dietary Zn concentration and floor type. P-values are presented for the main effect of dietary Zn concentration (Zn) and for the main effect of floor type (Fl).

‡ Total liver weight may deviate, due to losses during liver collection in the slaughterhouse.

[a,b] Mean values within a row and main effect with unlike superscript letters were significantly different ($P<0.050$).

Table 6.13. Effect of dietary zinc concentration on metacarpi characteristics from slaughtered sows group housed on different floor types between d28 and d108 of gestation (n= 54 for left front claw and n= 36 for right front claw).

Metacarpi[*]	Dietary treatment[†]			Floor type		SEM	P[‡]	
	0	50	100	Concrete	Rubber		Zn	Fl
Left front claw								
Length (mm)	90.2	90.9	89.6	89.5	91.0	0.4	0.569	0.145
Narrow dimension (mm)[§]	19.7	20.5	19.9	20.1	20.1	0.1	0.099	0.464
Wide dimensions (mm)[§]	16.7	17.0	17.2	17.1	16.9	0.1	0.449	0.490
Weight (g)	43.4	46.7	43.5	43.4	45.7	0.7	0.297	0.233
Right front claw								
Length (mm)	88.9	89.1	88.7	88.5	89.3	0.3	0.940	0.260
Weight (g)[¶]	37.9	38.4	37.9	37.6	38.6	0.3	0.882	0.208

Zn, zinc; F, floor type.
[*] Average of metacarpus 3 and 4 for length, dimensions and weight.
[†] Dietary treatment is presented as 0, 50, or 100 mg Zn/kg supplemented to the diet during gestation and lactation.
[‡] There were no interactions between dietary Zn concentration and floor type. P-values are presented for the main effect of dietary Zn concentration (Zn) and for the main effect of floor type (Fl).
[§] Narrow and wide dimensions of average M3 and 4 of left front claw were determined according to Figure 1 in Combs *et al.* (1991).
[¶] Average metacarpus 3 and 4 of the right front claw were weighed after the warm water bath for 24h at 75 °C.
[a,b] Mean values within a row and main effect with unlike superscript letters were significantly different (P<0.050).

Claw quality characteristics

Claw lesion score

The non-supplemented sows had a higher (worse) mean heel horn erosion score compared to the 100 mg Zn/kg supplemented sows at d50 of the reproductive cycle (P=0.013) (Table 6.14). The non-supplemented sows had a lower (better) mean skin lesion score compared to the 50 mg Zn/kg supplemented sows (P=0.025) and tended to be lower (better) compared to the 100 mg Zn/kg supplemented sows (P=0.070) at d50. No differences between dietary treatment groups were found for the other types of claw lesions at d50 or d140 (P>0.050). As such, floor type did influence claw lesions scores (Bos *et al.*, unpublished data).

Claw conformation

Claw dimension measurements

The mean heel height was lower for the non-supplemented and 100 mg Zn/kg supplemented sows compared with the higher heel height for the 50 mg Zn/kg supplemented sows (Table 6.15).

Independent of dietary Zn concentration, the mean toe height and mean claw length of sows was lower and shorter for the rubber floor compared with the concrete floor (P=0.001 and P=0.038, respectively) at d50 (Table 6.15). The mean heel height tended to be lower for sows housed on the concrete floor type at d50 (P=0.056) and was lower for sows housed on the rubber floor type (P=0.040) at d140.

Table 6.14. Effect of dietary zinc concentration on mean claw lesion scores[*] at d50 and d140 from sows housed on different floor types during group housing and that were followed three reproductive cycles (n= 131).

Claw lesion type (mm)	Reproductive cycle[†]						SEM	P[‡]	
	d50			d140				d50	d140
Dietary treatment[§]	0	50	100	0	50	100			
Heel horn erosion	48.2[a]	47.3[ab]	44.3[b]	60.1	58.1	57.2	0.76	0.014	0.161
Heel/sole junction separation	41.8	39.4	40.0	53.5	52.1	50.6	0.71	0.257	0.238
White line separation	48.5	45.5	45.7	55.2	53.8	53.3	0.81	0.161	0.432
Skin lesions	18.9[a]	22.9[b]	22.2[ab]	26.3	26.5	26.6	0.65	0.019	0.994
Horizontal wall cracks[¶]	41.9	42.1	42.1	42.4	44.1	43.2	0.75	0.869	0.564
Vertical wall cracks	26.2	28.2	28.4	29.9	32.2	29.9	0.80	0.392	0.402
Overgrown claw	30.9	28.3	29.1	38.8	38.7	37.9	0.58	0.601	0.721
Overgrown dewclaw	34.5	34.1	35.6	41.0	40.9	41.0	0.79	0.931	0.817

[*] Mean claw lesion score (mm) is the average score per lesion type for all sows including front and hind claws, lateral and medial digits.
[†] Reproductive phase (gestation, d50 and lactation, d140) did influence scores for all claw lesion types, except for horizontal wall cracks (P=0.249).
[‡] There were no interactions between dietary Zn concentration and floor type. P-values are presented for the main effect of dietary Zn concentration (Zn) at d50 and d140 of the reproductive cycle.
[§] Dietary treatment is presented as 0, 50, or 100 mg Zn/kg supplemented to the diet during gestation and lactation.
[¶] The superficial reference point that was incised into the dorsal horn wall for horn growth and wear measurements did not influence the scores for horizontal wall cracks (P>0.100).
[a,b] Mean values within a row and main effect with unlike superscript letters were significantly different (P<0.050).

Claw morphology calculations

The mean claw volume was lower for non-supplemented sows and tended to be lower for the 100 mg Zn supplemented sows compared with 50 mg Zn/kg supplemented sows (P=0.034 and P=0.064, respectively). The mean toe:heel ratio was higher for the non-supplemented sows compared with the lower mean toe:heel ratio of the 50 mg Zn/kg supplemented sows (P=0.018). Distal toe angle, sole area, and claw horn size were not different (Table 6.15).

The mean distal toe angle, sole area, and toe: heel ratio were lower for sows housed on rubber floors than sows housed on the concrete floors at d50 (Table 6.15). Mean claw volume was lower for the

rubber floor type compared with the concrete floor type at d140 (P=0.014). The mean claw horn size was not different (Table 6.15).

Table 6.15. Effect of dietary zinc concentration on claw conformation at d50 and d140 from sows housed on different floor types during group housing and that were followed three reproductive cycles (n= 131).

Claw conformation*	Reproductive cycle†						SEM	P‡	
	d50			d140				d50	d140
Dietary treatment§	0	50	100	0	50	100			
Claw dimensions (mm)									
Sole (base) length	24.8	24.9	24.4	26.3	27.2	26.8	0.15	0.206	
Claw width	27.5	27.9	27.3	27.2	27.6	27.5	0.13	0.661	
Length dorsal border	43.1	42.5	42.7	48.3	48.1	48.2	0.15	0.903	
Diagonal claw length	55.0	54.9	55.2	59.5	59.4	59.3	0.20	0.559	
Toe height	35.4	35.3	35.1	36.2	35.9	36.3	0.16	0.710	
Heel height	8.0	8.2	8.3	11.2	12.2	11.1	0.18	0.011	
Claw length	50.2	50.1	50.1	52.0	51.5	52.2	0.20	0.905	
Claw calculations¶									
Distal toe angle (°)	56.7	57.3	56.5	49.7	49.3	50.2	0.36	0.883	
Sole area (mm^2)	1384	1400	1373	1418	1424	1441	9.84	0.970	
Claw volume (mm^3)	11091	11532	11540	15961	17248	15823	281.7	0.025	
Claw horn size (mm^2)	1520	1537	1515	1624	1641	1638	10.7	0.643	
Toe:heel ratio	3.9	3.8	3.7	3.0	2.9	3.0	0.06	0.024	
Floor type	Concrete	Rubber	Concrete	Rubber					
Claw dimensions (mm)									
Sole (base) length	25.2	24.2	26.8	26.7			0.12	0.212	0.991
Claw width	27.8	27.3	27.4	27.4			0.11	0.283	0.830
Length dorsal border	43.0	42.6	48.8	47.5			0.12	0.838	0.218
Diagonal claw length	55.3	54.8	60.0	58.7			0.16	0.873	0.384
Toe height	36.4[a]	34.1[b]	35.9	36.4			0.13	0.001	0.492
Heel height	7.7	8.6	12.1[a]	10.7[b]			0.15	0.056	0.040
Claw length	51.2[a]	49.1[b]	52.3	51.5			0.17	0.038	0.572
Claw calculations¶									
Distal toe angle (°)	58.6[a]	55.0[b]	48.5	51.1			0.29	0.043	0.159
Sole area (mm^2)	1430[a]	1344[b]	1441	1413			7.96	0.026	0.523
Claw volume (mm^3)	11136	11616	17429	15031			229.5	0.248	0.014
Claw horn size (mm^2)	1543	1502	1654	1612			8.61	0.330	0.322
Toe:heel ratio	4.1[a]	3.5[b]	2.9	3.1			0.06	0.021	0.747

* Mean claw conformation measurements and calculations (mm) is the average score measurement for all sows including front and hind claws, lateral and medial digits.

† Reproductive phase (gestation, d50 and lactation, d140) did influence claw conformation measurements, except for claw width (P=0.216).

‡ There were no interactions between dietary Zn concentration and floor type. P-values are presented for the main effect of dietary Zn concentration (Zn), and for the effect of floor type (Fl) at d50 and d140 of the reproductive cycle.

§ Dietary treatment is presented as 0, 50, or 100 mg Zn/kg supplemented to the diet during gestation and lactation.
¶ Claw calculations included distal toe angle (sine of the length of the dorsal border and toe height), sole area (claw length × claw width), claw volume (sole area × heel height), claw horn size (claw width × diagonal claw length), and toe:heel ratio (toe height : heel height) (Calabotta *et al.,* 1982; Vermunt and Greenough, 1995; Manske, 2002; Bradley, 2008; Van Amstel and Doherty, 2010).
[a,b] Mean values within a row and main effect with unlike superscript letters were significantly different ($P<0.050$).

Horn growth and wear

No differences were observed between dietary treatment groups for horn growth and wear (Table 6.16). As such, horn growth and wear were lower for sows housed on rubber at d50 compared with the concrete floor type. No differences were found for horn growth and wear between floor types at d140 (Table 6.16).

Net horn growth (horn growth minus wear) was not different between dietary treatment groups and between floor types at d50 and d140 (Table 6.16). Net horn growth differed between d50 (-4.2 mm, horn wear dominated) and d140 (+4.4 mm, horn growth dominated) ($P<0.001$).

Table 6.16. Effect of dietary Zn concentration on horn growth and wear* at d50 and d140 from sows housed on different floor types during group housing and that were followed three reproductive cycles (n= 131).

Claw conformation (mm)	Reproductive cycle						SEM	*P*†	
	d50			d140				d50	d140
Dietary treatment‡	0	50	100	0	50	100			
Horn growth	12.4	12.7	12.3	20.9	20.8	21.7	0.24	0.667	
Wear rate	16.0	17.0	16.9	16.4	16.5	17.2	0.27	0.281	
Net horn growth§	-3.8	-4.4	-4.7	4.4	4.3	4.4	0.25	0.554	
Floor type	Concrete		Rubber	Concrete		Rubber			
Horn growth	13.7[a]		11.3[b]	21.1		21.2	0.19	0.001	0.634
Wear rate	17.7[a]		15.7[b]	16.6		16.9	0.22	0.001	0.676
Net horn growth§	-4.1		-4.5	4.5		4.3	0.20	0.862	0.974

* Horn growth and wear (mm) was determined from both lateral and medial claw digits of the left front and right hind claws.
† There were no interactions between dietary Zn concentration and floor type. P-values are presented for the main effect of dietary Zn concentration (Zn), and for the effect of floor type (Fl) at d50 and d140 of the reproductive cycle.
‡ Dietary treatment is presented as 0, 50, or 100 mg Zn/kg supplemented to the diet during gestation and lactation.
§ Net horn growth is horn growth minus wear and represents the balance between horn growth and wear throughout the reproductive cycle.
[a,b] Mean values within a row and main effect with unlike superscript letters were significantly different ($P<0.050$).

Histological claw characteristics

No dietary Zn or floor treatment differences were found for the characteristics of the transverse horn wall and transverse heel horn (Table 6.17).

The distance between dermal papillae of the sagittal heel horn tended to be lower for the non-supplemented sows (P=0.081) and was lower for the 50 mg Zn/kg supplemented sows (P=0.003) compared with the 100 mg Zn/kg supplemented sows (Table 6.17). No differences were found between dietary treatment groups for number of dermal papillae per 1000 µm, width of the papillae, or length of the longest papillae of the sagittal heel horn (P>0.050). Independent of dietary Zn concentration, the length of the longest papillae of the sagittal heel horn tended to be lower and the distance between dermal papillae was lower for sows housed on rubber floors than sows housed on concrete floors (Table 6.17).

Mechanical horn characteristics

None of the mechanical abaxial horn wall characteristics were significantly affected by dietary Zn concentration or floor type treatments (Table 6.18). Young's modulus differed between 1 mm/min and 15 mm/min test loading velocities (P=0.010), tended to be different for maximal stress (P=0.074) and did not differ for yield stress (P=0.221), showing visco-elastic properties of the horn wall. Differences between the two horn wall samples per claw digit per sow for both test velocities were found for Young's modulus, yield stress and maximal stress (P<0.001).

Table 6.17. Effect of dietary zinc concentration on histological claw characteristics[*] from sows (n= 36) housed on different floor types during group housing after slaughter at the third reproductive cycle (n= 71 for transverse horn wall, n= 54 for sagittal heel horn, and n= 75 for transverse heel horn samples).

Histological characteristics	Dietary treatment[†]			Floor type		SEM	*P*[‡]	
	0	50	100	Concrete	Rubber		Zn	Fl
Transverse horn wall								
Dermal lamellae	6.6	7.4	7.0	6.9	7.0	0.2	0.462	0.766
Distance	153.7	139.9	145.5	146.3	146.2	4.6	0.557	0.858
Width	56.4	51.9	50.0	51.9	53.6	2.7	0.631	0.900
Length	233.3	222.2	201.4	215.7	221.9	7.6	0.346	0.767
Sagittal heel horn								
Dermal papillae	3.0	2.9	2.5	3.0	2.7	0.1	0.183	0.218
Distance	315.7^{ab}	282.6^{a}	390.3^{b}	340.4^{a}	315.1^{b}	11.5	0.004	0.039
Width	126.6	131.8	150.1	130.9	137.6	5.2	0.224	0.475
Length	500.0	461.3	443.7	537.7	423.6	27.1	0.630	0.051
Transverse heel horn								
Horn tubules	7.4	6.5	6.7	6.9	6.8	0.2	0.108	0.747

Zn, zinc; Fl, floor type.

[*] Dermal papillae/lamellae, number of dermal papillae/lamellae per 1000 µm, visible at their full width; Distance, distance between the axis lines of the papillae/lamellae at their base (µm); Width, width of the dermal component halfway and perpendicular to the dermal papillae/lamellae (µm); Length, length of the longest papillae measured from the top of the dermal papillae/lamellae to the origin at the base (µm); Horn tubules, heel horn tubules density expressed as number of horn tubules within a defined surface area of 1 mm^2. Horn tubules that were only partially visible from two of the four sides of the defined surface area were also included.

[†] There were no interactions between dietary Zn concentration and floor type. P-values are presented for the main effect of dietary Zn concentration (Zn) and for the main effect of floor type (Fl).

[‡] Dietary treatment is presented as 0, 50, or 100 mg Zn/kg supplemented to the diet during gestation and lactation.

[a,b] Mean values within a row and main effect with unlike superscript letters were significantly different ($P<0.050$).

Table 6.18. Effect of dietary zinc concentration on mechanical claw characteristics[*] from sows housed on different floor types during group housing after slaughter at the third reproductive cycle (n= 36).

Mechanical characteristics	Test velocity[†]						SEM	*P*[‡]	
	1 mm/min			15 mm/min				1	15
Dietary treatment[§]	0	50	100	0	50	100			
Young's modulus (MPa)	68.3	84.0	70.0	96.6	100.1	97.0	6.3	0.986	0.998
Yield stress	10.8	12.9	10.1	13.1	14.4	12.9	0.8	0.716	0.976
Maximum stress	14.8	18.7	15.1	19.5	21.3	19.7	1.1	0.962	0.956
Floor type	Rubber		Concrete	Rubber		Concrete			
Young's modulus (MPa)	75.8		72.2	94.5		101.1	5.3	0.737	0.733
Yield stress	11.6		10.9	13.3		13.6	0.7	0.681	0.759
Maximum stress	17.0		15.3	20.3		20.0	0.9	0.980	0.957

[*] Young's modulus is a measure for the rigidity and stiffness of the horn, yield stress represents the point on the stress-strain diagram in which the material starts to lose its mechanical function and material properties starts to change at further loading, and maximal stress represents the maximum compression (Franck *et al.*, 2006).

[†] Mechanical claw characteristics were tested on two test velocity of the right front claw, 1 and 15 mm/min, to test if the abaxial horn wall had visco-elasticic properties. The abaxial horn wall does have these properties, because test velocities differ ($P<0.050$).

[‡] There were no interactions between dietary Zn concentration and floor type. P-values are presented for the main effect of dietary Zn concentration (Zn) and for the main effect of floor type (Fl) at test velocity 1 mm/min (1) and 15 mm/min (15).

[§] Dietary treatment is presented as 0, 50, or 100 mg Zn/kg supplemented to the diet during gestation and lactation.

[a,b] Mean values within a row and main effect with unlike superscript letters were significantly different ($P<0.050$).

Discussion

Dietary Zn concentration

Zinc status assessment and performance

Despite the considerable range in dietary Zn concentration among the treatments in the present study, the serum MT concentrations and concentrations of Zn in blood plasma, liver, bone, and horn wall did not respond to increased Zn inclusion levels in the diet. The lack of impact of Zn supplementation may have been (partly) attributed to the unintendedly higher analysed Zn concentration of the non-supplemented lactation diet (Zn from ingredients only). This concentration deviated considerably from what was formulated to an extent that all sows received Zn above the NRC requirements during the last week of gestation until weaning (d108-d143). Although sows received Zn above NRC requirements during this period, these results still indicate that the tested dietary range did not disturb Zn homeostasis, including the non-supplemented sows during gestation based on earlier studies in rats (Windisch and Kirchgessner, 1994a,b,c,1995b) and the

tightly regulated flow of Zn to ensure homeostasis. Maintaining Zn homeostasis is very important to ensure that all body processes are optimally regulated. Therefore, adjustments in the processes of Zn absorption and excretion may occur and have been shown in rats and weaned piglets (Weigand and Kirchgessner, 1980; Windisch and Kirchgessner, 1994a,c, 1995a; Martinez *et al.,* 2005; Jongbloed *et al.,* 2010). Although we did not measure absorption, it is likely that a higher Zn intake did not result in higher absorption and utilisation of Zn, due to the downregulation of the transport protein ZIP4 in the lumen (Martin *et al.,* 2013). Simultaneously, the amount of Zn in faeces might increase due to reduced absorption and increased excretion when the transport protein ZnT1 and MT are upregulated in the lumen (Martin *et al.,* 2013). Metallothionein binds Zn within the enterocyte and Zn will be excreted if the enterocyte is sloughed off (Martinez *et al.,* 2005; Jollif, 2011).

Most other studies found differences in plasma Zn (Hoekstra *et al.,* 1967; Hedges *et al.,* 1976; Kalinowski and Chavez 1984 and 1986) and liver and bone Zn concentration in sows (Hoekstra *et al.,* 1967; Hill *et al.,* 1983 a,b; Kalinowski and Chavez, 1991) and piglets (Prasad *et al.,* 1969; Revy *et al.,* 2002, 2004 and 2006; Schlegel *et al.,* 2010; Bikker *et al.,* 2011) fed diets with varying Zn concentration. Most of these studies, especially the studies on sows, compared the responses between a non-supplemented and supplemented treatment group without addition of phytase. The basal dietary Zn concentration without phytase was lower (<35 mg Zn/kg) compared to concentration of the basal diet in the present study during gestation with phytase (47 mg Zn/kg). Phytase was added in the present study to simulate practical conditions, but may have increased Zn status more than only Zn supplementation as found in piglets (Revy *et al.,* 2004, 2006; Schlegel *et al.,* 2010; Bikker *et al.,* 2011). In sows, however, addition of phytase did not affect the digestibility of Zn during gestation, but it did during lactation (Jongbloed *et al.,* 2013). The long-term impact of the addition of phytase on Zn status is unknown in sows during reproduction. Yet, the lower dietary Zn concentration of the other studies suggests that these non-supplemented sows were not able to maintain homeostasis and that those dietary Zn concentrations were insufficient. Further, it suggests that the dietary Zn concentrations with phytase were adequate for sows in the present study. This was also observed in rats where a lowered distribution of Zn between tissues, a decreased faecal Zn excretion, and decreased concentration of Zn in some tissues (digestive organs, plasma, hair, and skeleton) at insufficient dietary Zn concentrations was found (Windisch and Kirchgessner, 1994c). Differences between tissue Zn concentrations were negligible between adequate and excessive dietary Zn intake in the same study (Windisch and Kirchgessner, 1994c). At adequate and excessive dietary Zn concentrations, whole body Zn concentration seems not affected (Windisch and Kirchgessner, 1994a,b,c,1995b), indicating that Zn is redistributed between tissues and that this

redistribution is enhanced with increasing dietary Zn concentrations to protect tissues (Neathery *et al.,* 1973; Sunder *et al.,* 2008; Jongbloed *et al.,* 2010). This further supports that Zn homeostasis was maintained in the present study.

Liver and bone are major storage organs for Zn in pigs, and especially bone seems to accumulate Zn with increasing dietary Zn concentrations compared to other Zn status biomarkers, that reach a plateau at lower inclusion levels (Revy *et al.,* 2006; Bikker *et al.,* 2011). Possibly in sows, liver and bone will only accumulate Zn when dietary Zn intake is substantially beyond Zn requirements with or without addition of phytase, in an attempt to maintain Zn homeostasis. This suggests that in our study, the absorbed fraction was still at a level that did not require Zn accumulation in storage tissues. At excessive dietary Zn concentrations, Hill *et al.* (1983a,b) found a linearly increased liver Zn concentration in sows fed diets reaching pharmacological dietary Zn concentrations (>500 mg added Zn/kg diet). At these concentrations, the Zn absorption may have changed from a carrier mediated to a passive transport pathway (Martin *et al.,* 2013). As a response, Zn absorption and Zn (re)distribution between body tissues is disturbed, resulting in increased Zn concentration in the liver (Hill *et al.,* 1983a,b; Martin *et al.,* 2013). In the present study, because of the unintended higher concentration of Zn during lactation, it is possible that providing the sows with 129 mg Zn/kg diet during lactation ensured adequate Zn homeostasis also during gestation.

In the present study, sows' performance (BW, backfat thickness and BCS) and reproductive performance (average BW of weaned piglets) were already affected for the 100 mg Zn/kg supplemented sows. Hill *et al.* (1983a,b) observed a similar effect, but only at much higher levels (5000 mg added Zn/kg diet). Studies in cattle also found lower performance characteristics in their supplementation group (Fagari-Nobijari *et al.,* 2012; Dermauw *et al.,* 2015). Yet, it is not fully understood why performances would be lower during (excessive) Zn supplementation levels. Feed intake, Zn reserves and Zn requirements cannot explain the lowered performance characteristics in the present study, and no antagonistic effects of Cu, Ca and P were observed, which is in agreement with other studies (Hill *et al.,* 1983a,b; Martinez *et al.,* 2005). Furthermore, no noteworthy situations were reported throughout the experiment that could have influenced the observed results. Possibly, the protein expression in the liver and pancreas may be altered, which is related to cellular stress responses, transport, metabolism, and signal transduction, without influencing liver or pancreas Zn concentration and hepatic mRNA MT expression (Bondzio *et al.,* 2013; Pieper *et al.,* 2015). The lower performances of the piglets seem a logical result of the lower sow performances.

Despite the homeostatic reaction to dietary Zn within the tested range, plasma Zn and serum MT concentrations fluctuated during the reproductive cycle in the present study. This is in line with

observations in other sow studies (Hill *et al.,* 1983b; Kalinowksi *et al.,* 1984 and 1986; Girard *et al.,* 1996; van Riet *et al.,* 2015) and is in accordance with fluctuations of plasma Zn in women (Donangelo *et al.,* 2005) and sheep (Gürdoğan *et al.,* 2006).

Claw quality

In the present study, dietary Zn concentration showed minor effects on claw quality measurements, irrespective of floor type. This contradicts with improved claw lesion scores found in other studies in sows, showing a positive effect of organic Zn, Cu, and Mn supplementation (Aae, 2008; Anil *et al.,* 2010a,b; Da Silva *et al.,* 2010; Anil, 2011).

In the present study, most sows had at least one claw lesion, but only the mean heel horn erosion score was better for the 100 mg Zn/kg supplemented sows compared with the non-supplemented sows at d50 of the reproductive cycle. It is, however, questionable whether the difference of 4.2 mm is relevant. Furthermore, the mean lesion score was close to the upper threshold of 40 mm for healthy claws, suggesting that the overall good claw health might have overruled potential influence of Zn although effects of floor type were observed. It is remarkable that the non-supplemented sows were able to maintain claw quality at the level of the supplemented sows, without even differing in Zn status. The high Zn supply during lactation may have added to the potential of the sow to rule out Zn-induced changes, because responses to insufficient or excessive dietary Zn concentrations are expected within days or weeks as found in rats and piglets (Windisch and Kirchgessner, 1994a; Reeves *et al.,* 1995; Martinez *et al.,* 2005). Still, the non-supplemented group had an overall dietary Zn level well below commercial practices, without negative results.

Other factors may have also interfered with the results. Differences in claw quality measurements were observed only at d50 or only at d140 of the reproductive cycle, suggesting that the reproductive cycle is as important for claw quality as it is for Zn status. This is supported by the substantial fluctuation in net horn growth (horn growth minus wear) between gestation (-4.2 mm between d140 and d50, horn wear dominant) and lactation (+4.4 mm between d50 and d140, horn growth dominant). Furthermore, it is suggested that a lack of effect of dietary Zn on claw quality may be related to the study's duration being shorter than 12 months, which is related to the time required for the horn capsule to be produced for cattle (Hedges *et al.,* 2001; Griffiths *et al.,* 2007; Toni *et al.,* 2007; Lethbridge, 2009). The study duration of the present study was 14.8 months and, based on the length of the dorsal border and horn growth, the horn capsule in sows may have been produced in 5 to 6 months. Therefore, it seems that study duration may not have been the major factor influencing the lack of response in the present study. A final interfering factor is the Zn supplement used. In the present study, inorganic Zn was partly substituted by an organic Zn source

to simulate commercial conditions, and no major effects on claw quality were found. Other studies in sows found better claw lesion scores using exclusively organic Zn in a multiple mineral supplement, including Cu and Mn, compared with inorganic Zn, Cu, and Mn sources (Aae, 2008; Anil *et al.*, 2010a,b; Da Silva *et al.*, 2010; Anil, 2011). Possibly, Zn alone does not trigger the processes required to optimise claw quality and other minerals or dietary components are required as well.

Thus, dietary Zn concentration did not have a major impact on claw quality, which questions whether the dietary challenge was sufficiently large to induce detectable differences. This challenge seems appropriate, because floor type during group housing influenced claw quality measurements.

Floor type

Zinc status assessment and performance

Floor type did not influence performance characteristics and Zn status biomarkers, except for serum MT concentration. This lack of influence on Zn status biomarkers seems logical, because floor type is an external predisposing factor for claw lesions and lameness and therefore influences claw quality from the outside of the claw (Lethbridge, 2009). It remains unclear how floor type could have influenced the serum MT concentration.

Claw quality

The effect of floor type on claw quality measurements in the present study appeared to be greater than the effect of dietary Zn concentration and no interactions between floor type and dietary Zn concentrations were found. Sows housed on a rubber floor had better scores for some claw lesion types but worse scores for vertical horn wall cracks at d50 of gestation (data not shown). At d140, white line and claw length had better scores for sows housed on a rubber floor during gestation (data not shown). Other claw lesion types did not differ between floor types. This contradicts with another study in sows that found an increased risk of bad scores for toe overgrowth, heel sole cracks, and horn wall cracks in first parity sows housed on rubber slat mats as compared to sows housed on a concrete slatted floor (Calderón-Díaz, *et al.*, 2013). In the second parity of that study, sows housed on rubber slat mats had an increased risk of bad scores for toe overgrowth, heel sole cracks, and white line damage (Calderón-Díaz, *et al.*, 2013).

Discrepancies between studies may relate to the proportion of solid floor area, whereas a fully slatted floor was used by Calderón-Díaz, *et al.* (2013). Also the quality of the floor, characterised by the slip-resistance, abrasiveness, hardness, wear resistance, and age of the floor (Pluym *et al.*, 2013a), may explain the different results. Rubber slat mats might have been less abrasive

(Telezhenko *et al.,* 2008; Calderón-Díaz, *et al.,* 2013) and therefore may be softer than concrete slats (Mouttotou *et al.,* 1999; Scott *et al.,* 2006; Gillman *et al.,* 2009; KilBride *et al.,* 2009a,b; Calderón-Díaz, *et al.,* 2013). The influence of floor type on claw conformation in the present study might be a result of this softer rubber floor. Softer floors likely reduce natural horn wear (McKee and Dumelow, 1995; Kremer *et al.,* 2007; Platz *et al.,* 2007; Pluym *et al.,* 2013a), which was also found in the present study in which sows housed on rubber floors had less horn wear at d50 compared with sows housed on the concrete floor type. However, some claw lesions scores were better on rubber. It seems possible that a higher risk for claw lesions does not depend on insufficient wear alone, but rather on the balance between horn growth and wear and the load bearing (*e.g.* the pressure the floor exerts on the claw and how the load is distributed over the weight-bearing surface) may also be important factors (Winkler, 2005). In the present study, horn growth was lower for sows housed on rubber floors at d50 and the net horn growth was not affected, indicating that the balance between horn growth and wear could be maintained and that therefore claws were less prone to develop claw lesions. Similarly, the distal toe angle was lower on rubber floors, which indicates that there was less wear, less growth and a smaller sole area (Kroneman *et al.,* 1992). The load bearing may also be improved in the sows housed on rubber floors in the present study, because the histological claw characteristics showed a shortened distance between dermal papillae in the sagittal heel horn, indicating that the structure is stronger.

Furthermore, the slats properties are important (Webb, 1984; Boon and Wray, 1989; Anil *et al.,* 2007; Pluym *et al.,* 2013a). The rubber floor in the present study was attached to the concrete floor in a similar way as in the study of Calderón-Díaz, *et al.* (2013). One advantage of the rubber may be that the claws were less prone to being entrapped between the slats, but an important disadvantage was that the manure could not pass the slats easily (Calderón-Díaz, *et al.,* 2013). It has been reported that hygiene, in particular wet floors covered with liquid and manure, softens and irritates the claw and consequently decreases claw strength (Pluym *et al.,* 2013a). This seemed not the case in the present study in which the moisture content of the horn wall and horn wall strength were not affected.

Conclusion

The concentration of Zn status biomarkers was similar between dietary treatment groups and fluctuated throughout the reproductive cycle, indicating that Zn homeostasis was maintained. No interaction effects between dietary Zn concentration and floor type were found for Zn status biomarkers and claw quality measurements. Dietary Zn concentration had only minor influences on claw quality in sows, irrespective of floor type, whereas floor type affected multiple claw quality

measurements more positively. Differences between lateral and medial claw digits and between front and hind claws were observed. The reproductive phase had an important effect on the measured variables for Zn status and claw quality. The rubber top-layer seems to be more important in preventing claw lesions and improving claw quality than supplementing dietary Zn within the range used in the present study. Predominantly, the rubber top-layer was positively associated with claw quality than the concrete floors. Future studies should be conducted with dietary Zn concentrations in the non-supplemented lactation diet being lower and similar to the Zn concentrations in the non-supplemented gestation diet.

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Supplemental information

Observed differences between parity, claw digits (lateral and medial), and claws (front and hind) in sows related to claw quality measurement will be presented below.

Parity

Claw lesions score

Parity had no effect on heel horn erosion scores (P=0.434), but tended to influence separations along the heel-sole junction (P=0.099) in which sows in their third parity showed worse scores compared with the first parity. Parity did influence scores for separations along the white line (P<0.001), skin lesions scores (P<0.001), horizontal wall cracks (P<0.001), overgrown claw length (P<0.001), and overgrown dewclaw length (P<0.001) with sows in their third parity showing worse scores. Vertical wall cracks scores were worse for sows in their third parity compared with the first parity but better than the second parity (P<0.001).

Claw conformation

Claw dimension measurements. Base (sole) length was longer for sows in their third parity compared with the first parity but shorter than the second parity (P<0.001). The length of the dorsal border and claw length longer were longer sows in their third parity compared with the first parity (P<0.001 for both variables). Toe height was higher for sows in their third parity compared with the second parity (P<0.001). Diagonal claw length was longer (P<0.001) and heel height higher (P<0.001) for the third parity.

Claw morphology calculations. Distal toe angle was lower for sows in their third parity compared with the first parity, but a higher distal toe angle compared with the second parity (P<0.001). Sole area and toe:heel ratio were greater in the third parity compared with the first parity (P<0.001 and P=0.005, respectively). Claw volume and claw horn size was greater in the third parity (P<0.001 for both variables).

Horn growth and wear

Horn growth was lower for sows in their third parity compared with the first parity (P<0.001). Wear was lower for sows in their third parity compared with the first and second parity (P<0.001). Net horn growth (horn growth minus wear) was influenced by parity (P<0.001), in which sows in their third parity had a higher net horn growth compared with the second parity.

Claw digits

Claw lesion score

Later claw digits had a worse score for all types of claw lesion compared with the medial claw digits: heel horn erosion (+ 21.7 mm, $P<0.001$), separations along the heel sole junction (+14.6 mm, $P<0.001$) and white line (+22.8 mm, $P<0.001$), skin lesion scores (+7.6 mm, $P<0.001$), horizontal wall cracks (+6.2 mm, $P<0.001$), vertical wall cracks (+10.1 mm, $P<0.001$), overgrown claw length (+7.7 mm, $P<0.001$), and overgrown dewclaw length (+4.9 mm, $P<0.001$) (Table 6.19).

Claw conformation

Claw dimension measurements. All claw dimension measurements had higher values for the lateral claw digits. Later claw digits had a longer sole (base) length (+3.1 mm, P<0.001), a wider claw width (-3.3 mm, P<0.001), a longer dorsal border length (+2.1 mm, P<0.001), a longer diagonal claw length (+3.3 mm, P<0.001), higher toe height (+2.6 mm, P<0.001), higher heel height (+2.4 mm, P<0.001), and longer claw length (+1.8 mm, P<0.001) compared with the medial claw digits (Table 6.19).

Claw morphology calculations. Later claw digits had a higher distal toe angle (+1.9°, P<0.001), a greater sole area (+216.6 mm2, P<0.001), a greater claw volume (+5356 mm3, P<0.001) and a greater claw horn size (+272.4 mm2, P<0.001) compared with the medial claw digits (Table 6.19). The lateral claw digits had a lower toe:heel ratio (-0.1, P<0.071) compared with the medial claw digits.

Horn growth and wear

Horn growth and wear were higher for the lateral claw digits (+0.6 mm and +1.2 mm respectively, $P<0.001$) compared with the medial claw digits. Net horn growth was lower for the lateral than for the medial claw digits (-0.6 mm, $P<0.001$). The net horn growth of the lateral claw digits was negative and of the medial claw digits positive (Table 6.19).

Histological claw characteristics

Transverse horn wall. No significant differences were found between lateral and medial claw digits for the number of dermal lamellae per 1000 µm (P=0.887), distance between lamellae (P=0.821), width of the lamellae (P=0.267), or length of the longest lamellae of the transverse horn wall (P=0.228, Table 6.20).

Sagittal heel horn. No significant differences were found between lateral and medial claw digits for the number of dermal papillae per 1000 µm (P=0.808), distance between papillae (P=0.881), width

of the papillae (P=0.357), or length of the longest papillae of the sagittal heel horn (P=0.472, Table 6.20).

Transverse heel horn. The density of the heel horn tubules of the transverse heel horn, expressed as the number of horn tubules within a defined surface area of 1 mm2, was lower for the lateral digits compared with the medial digits (P=0.033, Table 6.20).

Mechanical claw characteristics

Abaxial horn wall was thicker for the lateral claw digits compared with the medial claw digits (*P*<0.001). Young's Modulus, yield stress and maximum stress of 1 mm/min test velocity did not differ between the lateral and medial digit of the right front claw (*P*=0.183, *P*=0.361, *P*=0.101, respectively). Young's Modulus, yield stress and maximum stress of 15 mm/min test velocity did not differ between the lateral and medial digit of the right front claw (*P*=0.110, *P*=0.930, *P*=0.496, respectively) (Table 6.20).

Claw

Claw lesion score

Hind claws had worse score for heel horn erosion (+ 3.2 mm, *P*<0.001), separations along the heel sole junction (+3.9 mm, *P*<0.001) and white line (+1.7 mm, *P*=0.001), skin lesion scores (+1.0 mm, *P*=0.021), and vertical wall cracks (+3.8 mm, *P*<0.001) compared with the front claws (Table 6.19). Hind claws had a better score for horizontal wall cracks lesion score (-1.1 mm, *P*=0.045), overgrown claw length (-1.3 mm, *P*<0.001), and overgrown dewclaw length (-17.6 mm, *P*<0.001) compared with the front claws (Table 6.19).

Claw conformation

Claw dimension measurements. Hind claws had a longer dorsal border length (+1.2 mm, P<0.001), and higher toe height (+0.5 mm, P<0.001) compared with the front claws. Hind claws had a shorter sole (base) length (-1.8 mm, P<0.001), a narrower claw width (-3.8 mm, P<0.001), shorter diagonal claw length (-3.5 mm, P<0.001), lower heel height (-2.3 mm, P<0.001), and a shorter claw length (-2.5mm, P<0.001) compared with the front claws (Table 6.19).

Claw morphology calculations. Hind claws had a higher toe:heel ratio (+0.4, P<0.001) compared with the front claws. Hind claws had a lower distal toe angle (-1.0°, P<0.001), a smaller sole area (-264.1 mm^2, P<0.001), a smaller claw volume (-5664 mm^3, P<0.001) and a smaller claw horn size (-311.2 mm^2, P<0.001) compared with the front claws (Table 6.19).

Horn growth and wear

Horn growth and wear were higher for the hind claws (+2.1 mm and +2.3 mm respectively, $P<0.001$) compared with the front claws. Net horn growth was not different between front and hind claws ($P=0.225$, Table 6.19).

Table 6.19. Differences in claw quality between lateral and medial claw digits in sows followed for three reproductive cycles (n= 131).

Claw quality measurement	Claw digit		Claw		SEM	*P*	
	Medial	Lateral	Front	Hind		Digit	Claw
Claw lesion type (mm)[*]							
Heel horn erosion	41.4	63.1	50.7	53.9	0.43	<0.001	<0.001
Heel/sole junction separation	38.7	53.3	44.1	47.9	0.41	<0.001	<0.001
White line separation	38.8	61.6	49.3	51.1	0.44	<0.001	0.001
Skin lesions	20.0	27.6	23.3	24.3	0.37	<0.001	0.021
Horizontal wall cracks	39.5	45.7	43.1	42.1	0.43	<0.001	0.045
Vertical wall cracks	24.0	34.1	27.2	31.0	0.45	<0.001	<0.001
Overgrown claw	29.9	37.6	34.4	33.2	0.34	<0.001	<0.001
Overgrown dewclaw	35.3	40.2	46.5	29.0	0.44	<0.001	<0.001
Claw dimensions (mm)[†]							
Sole (base) length	24.1	27.2	26.6	24.8	0.09	<0.001	<0.001
Claw width	25.8	29.1	29.4	25.6	0.07	<0.001	<0.001
Length of dorsal border	44.4	46.4	44.8	46.0	0.10	<0.001	<0.001
Diagonal claw length	55.5	58.8	58.9	55.4	0.12	<0.001	<0.001
Toe height	34.4	37.0	35.4	35.9	0.09	<0.001	<0.001
Heel height	8.6	11.0	11.0	8.7	0.11	<0.001	<0.001
Claw length	50.1	51.9	52.3	49.7	0.12	<0.001	<0.001
Claw calculations[‡]							
Distal toe angle (°)	52.4	54.3	53.7	53.0	0.22	<0.001	<0.001
Sole area (mm^2)	1298	1515	1539	1275	5.15	<0.001	<0.001
Claw volume (mm^3)	11177	16534	16706	11017	161.4	<0.001	<0.001
Claw horn size (mm^2)	1441	1713	1733	1421	5.53	<0.001	<0.001
Toe:heel ratio	3.4	3.3	3.2	3.5	0.04	0.071	<0.001
Horn gorwth and wear (mm)[§]							
Horn growth	16.1	16.5	14.9	17.6	0.19	<0.001	<0.001
Wear rate	16.1	17.3	15.5	17.8	0.15	<0.001	<0.001
Nethorn growth	0.02	-0.8	-0.6	-0.2	0.20	<0.001	0.225

Digit, differences between lateral and medial claw digits; Claw, differences between front and hind claws.

[*] Mean claw lesion score (mm) is the average score per lesion type for all sows for lateral and medial claw digits and for front and hind claws.

[†] Mean claw conformation measurements and calculations (mm) is the average score measurement for all sows for lateral and medial claw digits and for front and hind claws.

[‡] Claw calculations included distal toe angle (sine of the length of the dorsal border and toe height), sole area (claw length × claw width), claw volume (sole area × heel height), claw horn size (claw width × diagonal claw length), and toe:heel ratio (toe height : heel height) (Calabotta *et al.*, 1982; Vermunt and Greenough, 1995; Manske, 2002; Bradley, 2008; Van Amstel and Doherty, 2010).

[§] Horn growth and wear (mm) was determined from both lateral and medial claw digits of the left front and right hind claws. Net horn growth is horn growth minus wear and represents the balance between horn growth and wear throughout the reproductive cycle.

Table 6.20. Differences in histological and mechanical claw characteristics between lateral and medial claw digits in sows (n= 36) after slaughter at the third reproductive cycle (n= 71 for transverse horn wall, n= 54 for sagittal heel horn, and n= 75 for transverse heel horn samples).

Claw quality measurement[*]	Claw digit		SEM	*P*
	Medial	Lateral		
Histological claw characteristics[†]				
Transverse horn wall				
Dermal lamellae	7.0	7.0	0.2	0.887
Distance	146.8	145.7	4.6	0.821
Width	54.9	50.5	2.7	0.267
Length	208.9	228.9	7.6	0.228
Sagittal heel horn				
Dermal papillae	2.8	2.8	0.1	0.808
Distance	322.1	329.3	11.5	0.881
Width	139.8	129.1	5.2	0.357
Length	490.6	447.1	27.1	0.472
Transverse heel horn				
Horn tubules	7.2	6.5	0.2	0.033
Mechanical claw characteristics[‡ §]				
Test velocity, 1 mm/min				
Young's modulus (MPa)	79.3	68.6	4.3	0.183
Yield stress	11.6	10.9	0.7	0.361
Maximum stress	16.5	15.8	0.9	0.101
Test velocity,15 mm/min				
Young's modulus (MPa)	102.7	93.0	3.6	0.110
Yield stress	13.5	13.5	0.4	0.930
Maximum stress	19.9	20.4	0.5	0.496

[*] Histological claw characteristics determined for both front claws, mechanical claw characteristics determined for the right front claw.

[†] Dermal papillae/lamellae, number of dermal papillae/lamellae per 1000 µm, visible at their full width; Distance, distance between the axis lines of the papillae/lamellae at their base (µm); Width, width of the dermal component halfway and perpendicular to the dermal papillae/lamellae (µm); Length, length of the longest papillae measured from the top of the dermal papillae/lamellae to the origin at the base (µm); Horn tubules, heel horn tubules density expressed as number of horn tubules within a defined surface area of 1 mm^2. Horn tubules that were only partially visible from two of the four sides of the defined surface area were also included.

[‡] Young's modulus is a measure for the rigidity and stiffness of the horn, yield stress represents the point on the stress-strain diagram in which the material starts to lose its mechanical function and material properties starts to change at further loading, and maximal stress represents the maximum compression (Franck *et al.,* 2006).

[§] Mechanical claw characteristics were tested on two test velocity, 1 and 15 mm/min, to test if the abaxial horn wall had visco-elasticic properties. The abaxial horn wall does have these properties, because test velocities differ ($P<0.050$).

Chapter 7

General Discussion

Claw lesions in sows are an important multifactorial disorder, resulting in negative consequences for animal welfare and farm profitability. Malnutrition has been reported as one of the factors that predispose to a deteriorated claw quality. Zinc as essential micromineral is involved in horn production, together with sulphur-containing amino acids and biotin, although its specific role is inconclusive. This thesis focused on Zn status assessment and the role of Zn in claw quality in pigs (Figure 7.1).

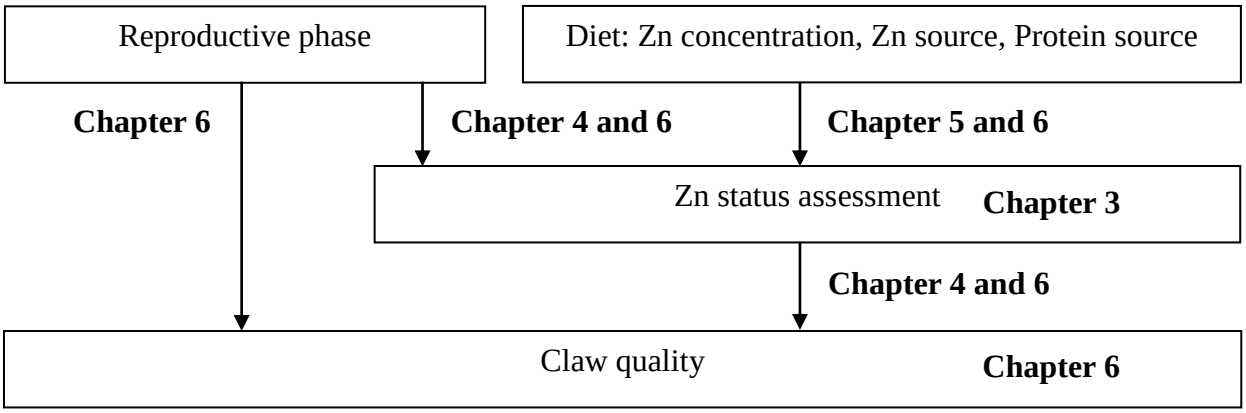

Figure 7.1. Schematic presentation of thesis objectives to evaluate the role of dietary Zn in claw quality and Zn status assessment in pigs, specifically to: 1) evaluate the suitability of Zn status biomarkers for Zn status assessment in production animals (Chapter 3); 2) determine possible fluctuations of Zn status biomarkers throughout a reproductive cycle in sows (Chapter 4); 3) determine the effect of protein and Zn source on Zn status and Zn bioavailability in sows (Chapter 5); and 4) determine the effect of dietary Zn concentration on Zn status biomarkers and claw quality in weaned piglets and in sows (Chapter 6).

Zinc status: assessment, reproduction and dietary Zn concentration

Do zinc status biomarkers fluctuate throughout the reproductive cycle?

The results from several chapters (4, 5 and 6) show that Zn status biomarkers fluctuate throughout a reproductive cycle in sows, independent of dietary Zn concentration and source. The pattern of fluctuation differed between biomarkers.

Only few studies in sows have reported the fluctuations of plasma Zn concentration throughout the reproductive cycle (Hoekstra *et al.*, 1967; Palludan and Wegger, 1976; Kalinowski and Chavez, 1984, 1986; Girard *et al.*, 1996; Richards, 1999). Even fewer studies included plasma alkaline phosphatase (ALP) concentrations (Hoekstra *et al.*, 1967; Kalinowski and Chavez, 1984, 1986), although the suitability is questionable according to Chapter 3. Fluctuations of serum metallothionein (MT) concentration are not reported at all. Further, the limited data available showed contradictory results during both gestation and lactation: some studies found no significant fluctuation of plasma Zn and ALP concentrations, whereas others found fluctuations in function of

parity and dietary Zn concentration (Table 7.1). Different experimental designs may have partly influenced the discrepancies found between studies.

The results of this thesis suggest that the observed fluctuations are more related to physiological adjustments to maintain Zn homeostasis rather than parity or dietary Zn concentration. In women, an anabolic phase during early pregnancy and a catabolic phase at the end of pregnancy have been suggested (Lain and Catalano, 2007). In sows, Jongbloed *et al.* (2010) also reported this gestation anabolism, in which a gravid sow retains more Zn than a non-gravid sow. Therefore, increased or high plasma Zn concentrations may reflect the anabolic phase, especially after weaning, whereas a decreased or low plasma Zn concentration at the end of gestation and lactation may reflect the catabolic phase. The catabolic phase, with low plasma Zn concentrations, corresponds to the exponential increase of Zn deposited in the foetus (Palludan and Wegger, 1976) and Zn required for milk synthesis. Indeed, the transfer of Zn to the foetus and new-born pig is considerable (Papadopoulos *et al.,* 2009; Matte *et al.,* 2014), in contrast with iron, for instance.

The extent to adjust physiologically may depend on Zn reserves and on the ability of a sow to adjust to maintain Zn homeostasis throughout reproduction. This may explain some differences between studies. A lack of fluctuation in plasma Zn concentration may indicate that a sow is able to maintain Zn homeostasis or that her Zn requirements are met, whereas a decrease reflects a temporarily altered Zn metabolism. This decrease is temporary, because plasma Zn concentration rises again at the end of lactation, as revealed in chapters 4 and 6b. Fluctuations of plasma Zn concentrations are expected in Zn deficient sows having little Zn reserves, as fluctuations were found in sows provided a diet with 4-5 mg Zn/kg diet (Palludan and Wegger, 1976) or 10 mg Zn/kg diet (Kalinowski and Chavez, 1986). These sows are more challenged to maintain Zn homeostasis and fluctuations are required for Zn mobilisation and Zn (re)distribution.

The fluctuation of serum ALP concentration (Chapter 4) differed from the fluctuations of plasma Zn and serum MT concentration. Based on the regulatory function of ALP in bone to form hydroxyapatite crystals, serum ALP concentration may reflects bone metabolism rather than Zn status. This cannot be confirmed by results of other studies, which observed either fluctuations in plasma ALP or bone marker concentration (Hoekstra *et al.,* 1967; Kalinowski and Chavez 1984, 1986; Liesegang *et al.,* 2006).

Table 7.1. Fluctuations of zinc status biomarkers throughout the reproductive cycle in several sow studies.

Biomarker	Fluctuation*		Parity range	Reference
	Gestation†	Lactation†		
Plasma Zn	Decreased linearly after d0 and increased quadratically towards parturition	No significant fluctuations	1-11	Chapter 4
	Decreased in all treatment groups from d0-d50	Decreased in all treatment groups from d108-d143	1-3	Chapter 6b
	No significant fluctuations	No significant fluctuations	1	Hoekstra *et al.*, 1967
	Non-supplemented Zn group (n= 4): decreased, Zn-supplemented group (n= 1): decreased in during the last 2 weeks of gestation	Non-supplemented Zn group: increased until 1 week postpartum, then decreased until weaning (n= 4), Zn supplemented group (n= 1): increased	1	Palludan and Wegger, 1976
	Tendency to decreased concentrations for both treatment groups. Decrease more marked in non-supplemented Zn group	Tendency to decreased concentrations for both treatment groups. Decrease more marked in non-supplemented Zn group	4-7	Kalinowski and Chavez, 1984
	Non-supplemented Zn group: decreased to d83 and increased slightly towards parturition, Zn supplemented group: increased from d22-d53 and maintained higher than d22 until parturition	Non-supplemented Zn group: decreased, Zn-supplemented group: increased	1	Kalinowski and Chavez, 1986
	Decreased 10-15 weeks for primiparous sows, decreased continuously in 2nd parity sows	No significant fluctuations	1&2	Girard *et al.*, 1996
	No significant fluctuations	No significant fluctuations	1	Richards, 1999
Plasma ALP	Increased linearly after d0 and decreased quadratically towards parturition	No significant fluctuations	1-11	Chapter 4
	No distinct fluctuations. Concentrations higher as parturition approached compared with mid-gestation	No distinct fluctuations. Concentrations higher during lactation compared with mid-gestation	1	Hoekstra *et al.*, 1967
	Both treatment groups: slightly lower at d113 compared with d86	Lower at d7 and 14 postpartum compared with d86 of gestation for both treatment groups	4-7	Kalinowski and Chavez, 1984
	Non-supplemented Zn group: decreased, Zn-supplemented group: tendency to increased concentrations until d83, decreased at d113	Non-supplemented Zn group: constant low, Zn-supplemented group: decreased during d7 of lactation, increased slightly at d14	1	Kalinowski and Chavez, 1986

d, days of the reproductive cycle; n, number of sows; ALP, Alkaline phosphatase, determined in serum or plasma. *Fluctuations are presented per dietary treatment group (non-supplemented, Zn-supplemented, all sows). †Gestation represents d0 (insemination)- d115 (parturition) and lactation represents d116-d143 (weaning).

Dietary Zn concentration and the impact on Zn status

Throughout our studies, the observed fluctuations in Zn status biomarkers were independent of any dietary treatment. This is in agreement with the findings of Hill *et al.* (1983a,b) below pharmacological dietary Zn inclusion levels (<500 mg added Zn/kg) but in contrast with other studies that also used sows with adequate Zn stores before the dietary Zn intervention (Hoekstra *et al.,* 1967; Hedges *et al.,* 1976; Kalinowski and Chavez, 1984 and 1986). Plasma Zn concentration is the most widely used biomarker for Zn status. If plasma Zn concentrations of all studies on sows were plotted against the total dietary Zn concentration (Figure 7.2), the plasma Zn concentrations reported in most studies varies between 50 and 150 μg/dL, irrespective of dietary Zn concentration and composition (Hoekstra *et al.,* 1967; Hedges *et al.,* 1976; Kalinowski and Chavez, 1984 and 1986; Hill *et al.,* 1983a; Tremblay *et al.,* 1989; Wuryastuti *et al.,* 1991; Girard *et al.,* 1996).

This supports the idea that the plasma levels are a reflection of the equilibrium between absorption and excretion to maintain homeostasis (Buckley and D'Mello, 2000; King, 2000; King, 2011). Changes in dietary Zn intake causes adjustments in Zn metabolism, with absorbed Zn being (re)distributed among body tissue and fluids (King, 2000; McDowell, 2003). Figure 7.2 suggests that plasma Zn concentration does not respond drastically to changes in dietary Zn intake until its capacity to adjust is exceeded. Pharmacological dietary Zn concentrations (>551 mg Zn/kg) seem to increase plasma Zn concentration (Figure 7.2), whereas dietary Zn concentrations below estimated Zn requirements (non-supplemented, ±30-50 mg Zn/kg diet from ingredients) did not consistently lead to lower plasma Zn concentrations (below 50 μg/dL). Thus Zn in plasma is important as part of the tightly regulated Zn homeostasis controlled by absorption and excretion (King *et al.,* 2000; Buckley and D'Mello, 2000; Hill and Link, 2009). At insufficient dietary Zn concentrations (below Zn requirement, marginal), absorption is highly efficient and excretion of Zn low. That change to a lower absorption and higher excretion of Zn at increasing dietary Zn concentrations to maintain equilibrium (*i.e.* homeostasis) (Weigand and Krichgessner, 1980; Jongbloed *et al.,* 2010). At equilibrium, concentrations of Zn in body tissues are similar between adequate (between Zn requirement and maximum allowance of 150 mg Zn/kg) and excessive (above maximum allowance) dietary Zn concentrations but differ with concentrations at insufficient Zn levels as found in rats and piglets (Windisch and Kirchgessner, 1994c; Martinez *et al.,* 2005; Martin *et al.,* 2013; Pieper *et al.,* 2015).

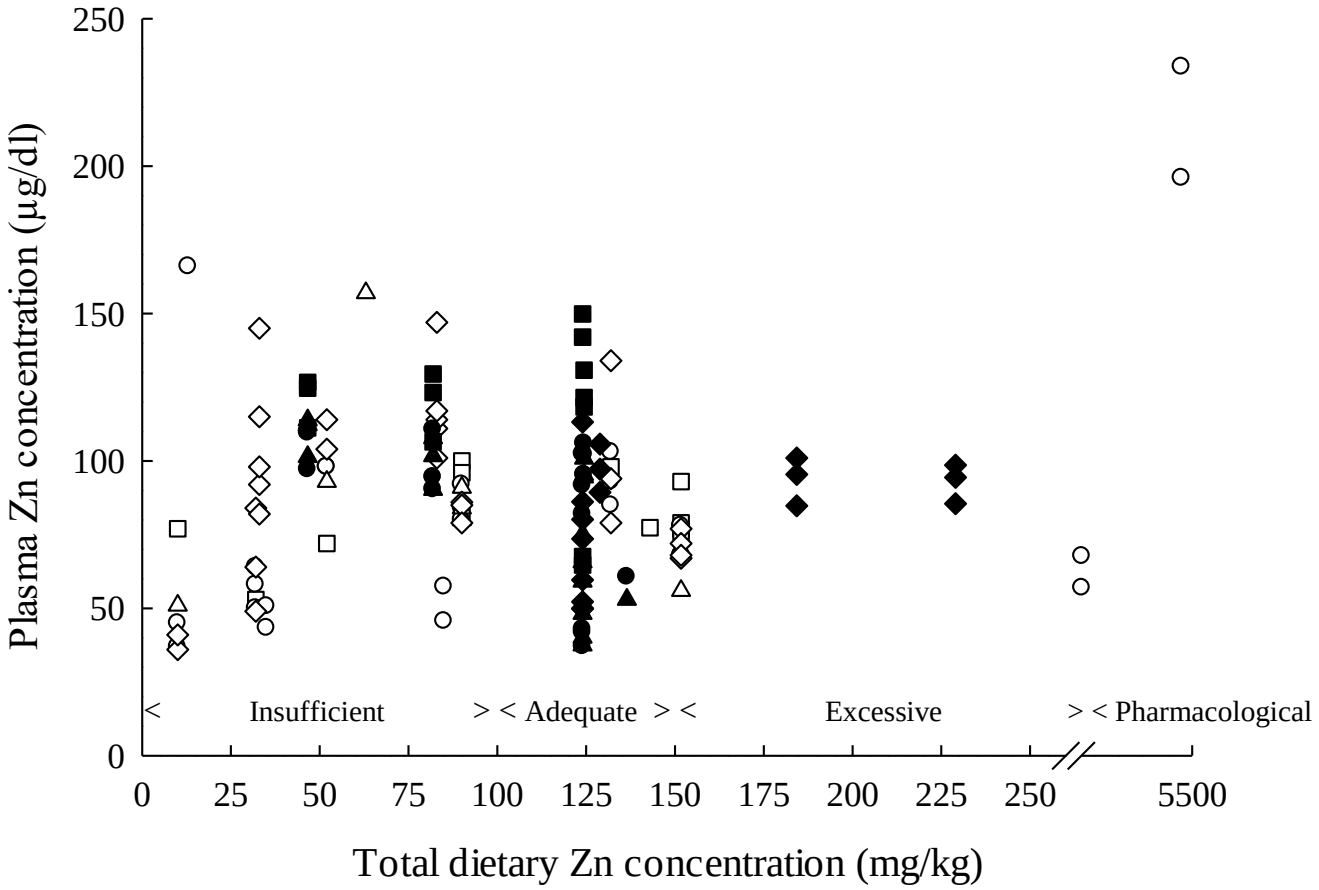

Figure 7.2. Plasma zinc concentrations (µg/dL) in sows based on the total dietary Zn concentration (ingredients and supplement) and reproductive phase. Values classified in early gestation (d0-d41, □), mid gestation (d42-d107, ○), end gestation (d108-d115, Δ) and lactation (d116-d143, ◊). Symbols without filling represent the plasma Zn concentrations of available literature (Hoekstra *et al.,* 1967; Hedges *et al.,* 1976; Kalinowski and Chavez, 1984 and 1986; Hill *et al.,* 1983a; Tremblay *et al.,* 1989; Wuryastuti *et al.,* 1991; Girard *et al.,* 1996). Symbols with black filling represent the plasma Zn concentrations of our studies (Chapter 4, 5 and 6b).

In non-supplemented weaned piglets, however, the capacity of plasma Zn concentration to adjust seemed exceeded (Chapter 6a). The duration of Zn deprivation as well as other dietary and animal related factors previously reported to influence Zn metabolism may influence the plasma Zn adjustments. These factors include high dietary Ca and Cu concentrations, addition of phytase, presence of phytate, Zn source, reproduction, production level, parity, and available Zn reserves (Revy *et al.,* 2004; Jongbloed *et al.,* 2010; Bikker *et al.,* 2011).

As Zn is tightly regulated through absorption and excretion and plasma Zn concentration only responds when its capacity is exceeded, it is questionable whether the methods used to determine Zn requirements are adequate to distinguish between responses observed at different dietary Zn

concentrations. Further consideration is required to examine whether the estimated Zn requirements meet the demands of all processes that require Zn, such as claw quality, immunity, and reproduction.

Are the estimated Zn requirements adequate or are the current methods inadequate?

Zinc inclusion in the diet of sows usually exceeds the estimated requirements (Table 7.2). The surplus of Zn will not be absorbed and excreted and this negatively effects the environment. Providing sows a diet with a low dietary Zn concentration does not necessarily mean that sows become Zn depleted or even Zn deficient. Based on our findings, sows are able to maintain plasma Zn concentration above 50 µg/dL when no Zn was supplemented during gestation (Chapter 6b), although results in chapter 4 show that concentrations below this level were found when Zn was supplemented to sows. Suttle (2010) suggests a marginal value between 40 and 60µg/dL for plasma Zn concentrations. Below that threshold, Zn depletion can develop. In this context, one may wonder whether the Zn requirements estimated in several evaluation systems (see Table 1.1) are adequate or that the biomarkers for Zn status assessment were inadequate. If sows require less Zn than estimated, dietary Zn concentration can be lowered, reducing the unabsorbed Zn fraction excreted to the environment. Surprisingly, the conceivably over-estimated requirements for sows are based on dated studies on a small number of sows as reported by Jongbloed *et al.* (2010). Furthermore, these studies used a non-supplemented control group and only one Zn supplementation group below 500 mg added Zn/kg. The responses of Zn status biomarkers at a wide range of dietary Zn concentrations are unknown, while the majority of the studies focus on specific effects of Zn availability or other dietary components, such as phytase and phytate.

Plasma Zn concentration as biomarker for Zn status has its limitations, because confounding factors, including stress, infection or disease, feeding state, reproductive phase, method of blood collection and concomitant blood sample transport, storage and analysis, may interfere with the concentrations observed (Delves, 1985; King, 1990; McDowell, 2003; Hess *et al.*, 2007; Hill and Link, 2009; Naithani *et al.*, 2014). These limitations hamper the specificity of plasma Zn concentration and in most studies other Zn status biomarkers were included in an attempt to overcome these limitations. In the longitudinal sow study (Chapter 6b), other Zn status biomarkers were included. However, these biomarkers showed unexpected responses to dietary Zn intake (Chapter 6b): liver and bone Zn concentration did not respond to increased dietary Zn concentration and sow performance as well as reproductive performance (*e.g.* average BW of weaned piglets (kg)) showed lower values for the 100 mg Zn/kg supplemented sows. These responses are (partly)

influenced by the high dietary Zn concentration during lactation and addition of phytase. Still, biomarkers used additionally to plasma Zn concentration in other studies (plasma ALP, bone and liver Zn concentrations of dam and progeny and reproductive performances) showed different responses between studies (Chapter 3).

Although there may be some debate on the use of adequate biomarkers for Zn status assessment, the fact that none of them was significantly affected, nor that most sows' reproductive performance variables and claw quality seemed affected, suggest that Zn requirements may be well below the maximal allowed concentration of 150 mg/kg.

Did experimental conditions limit our observations?

The observational and longitudinal study in this thesis (Chapter 4 and 6b) has offered new insights in the fluctuations of Zn biomarkers throughout the reproductive cycle and Zn status assessment in sows. However, a number of limitations need to be considered.

An important limitation of the observational study (Chapter 4) was that we used only one group of sows and followed them as one group for one reproductive cycle. Random effects such as genotype and environmental influences were therefore not taken into account. The observed fluctuations might have been different between groups of sows or between seasons. However, dietary Zn levels were well controlled and studies reported in chapter 4, 5 and 6b showed similar patterns in plasma Zn concentration during gestation. The influence of these random effects seems therefore unlikely.

Another limitation was that the sows in the longitudinal study (Chapter 6b) were not Zn depleted before the start of the experiment. This choice simulated practical conditions, indicating that their body Zn stores were likely adequate. In other studies, pigs had been Zn depleted before adding a dietary Zn supplement and differences between treatment groups were found (Wedekind *et al.*, 1994; Revy *et al.,* 2006; Bikker *et al.,* 2011). It can be questioned if using these pigs really reflects their long-term dietary zinc requirements. Therefore, we chose to perform a long-term study, assuming that this would be a more reliable indicator of the long-term Zn requirements of sows. Although the Zn concentrations of the lactation diets were higher than anticipated, our study still suggests that low dietary zinc levels during gestation may be satisfactory on the long term. Other studies used sows that were also not Zn-depleted before the start of the experiment and found differences between treatment groups (Kalinowski and Chavez, 1984 and 1986) or found only differences at pharmacological dietary Zn concentration (>500 mg added Zn/kg diet) and not at lower inclusion levels (non-supplemented, 50 and 500 mg added Zn/kg diet) from 30 kg bodyweight onwards until their second parity (Hill *et al.,* 1983a,b). Furthermore, previous studies

suggest that the capacity to store Zn which is readily available during low dietary Zn intake is limited (Buckley and D'Mello, 2000; McDowell, 2003), indicating that body Zn stores are readily depleted if insufficient dietary Zn is fed. These findings suggest that a Zn depletion period before the start of the study to deplete body Zn stores may not be essential.

In the longitudinal study (Chapter 6b), the dietary Zn concentration of the non-supplemented lactation diet was considerably higher than formulated and corresponded practical dietary Zn concentrations. This could have (partly) an impact on the potential influence of dietary Zn concentration. It did not affect plasma Zn concentrations and fluctuations, but may have interfered with the body tissue concentrations. The highest dietary Zn concentration during lactation may even have negatively affected (reproductive) performance.

Dietary Zn concentrations and claw quality

In our study, we found no major role of dietary Zn concentration on claw quality in sows, however, it seems to affect claw quality in weaned piglets.

Despite the importance of claw disorders in pigs, to date no studies have evaluated the effect of dietary Zn concentration. Few studies determined the effect of (partial) organic Zn sources combined with organic Cu and Mn supplementation on claw quality (mainly claw lesion scores) in sows (Table 7.2). These studies observed a decreased number of lesions or a similar lesion score compared with its previous evaluation. These findings differed between studies dependent on type and severity of claw lesions, and differed within studies between the herds included. Another study found no effect of concentration and source of Zn, Cu and Mn on claw conformation (Bradley, 2010). Despite the observed effects, these results do not illustrate the possible critical role of Zn in maintaining claw quality in sows, because the possible effect of Zn cannot be distinguished from the combined effect of Zn, Cu, and Mn. Studies in ruminants showed also contradictory responses (Table 7.2). This suggests that claw quality is less responsive to Zn supplementation compared with insufficient dietary Zn concentrations (below requirements) that can evolve into (marginal) Zn deficiency or that publication is biased, publishing only positive impacts of dietary Zn concentration.

Important interfering factors, such as study duration, have been reported in literature, which may have influenced the response of dietary Zn concentration on claw quality. Study duration is a critical argument, because the horn capsule grows slowly. In cattle, this capsule is produced in 12 to 30 months and changes may be seen only after at least 12 months (Griffiths *et al.,* 2007; Lethbridge, 2009). However, claw conformation differs between cattle and pigs (Kroneman *et al.,* 1992). Based

on our measurements (Chapter 6a and 6b), the horn capsule seems to be renewed in 2 months in weaned piglets and between 5 and 9 months in sows. Therefore, study duration may not be the major interfering factor in pigs. The study duration in sows was 14.8 months and no major differences in claw quality were found (Chapter 6b). On the contrary, differences in claw conformation in weaned piglets were observed even within the 5-week experimental period (Chapter 6a). This suggests that age and its concomitant body reserves are more important than study duration, because piglets as well as calves with a dorsal horn growth of 4.9-7.5 mm/month have a faster horn growth (Vermunt and Greenough, 1995; Winkler, 2005). Potentially, Zn may be more important for claw quality in gilts before their first insemination than older sows.

Furthermore, claw quality in non-supplemented sows was quite similar compared with Zn-supplemented sows and plasma Zn concentrations showed no differences between treatment groups (Chapter 6b), suggesting that the animal could absorb the amount of Zn required to maintain claw quality. The body Zn stores of these sows were adequate and there were no indications that the non-supplemented Zn group was Zn deficient. The opposite may be true for weaned piglets, because plasma Zn concentration was lower for the non-supplemented group compared with the Zn-supplemented group and showed differences in claw conformation (Chapter 6a). Therefore, weaned piglets seem to require higher dietary Zn concentrations to maintain claw quality compared with sows. Yet, we found no indications that the higher Zn requirements to maintain claw quality in weaned piglets was related to an interrupted or inefficient diffuse nutrient supply (required for keratinisation) from the dermis to the avascular epidermis, as stated by Tomlinson *et al.* (2004) and Muelling (2009). The histological claw characteristics did not differ between the non-supplemented and the Zn-supplemented piglets, whereas it differed between treatment groups in sows. Potentially, the presence of the total nutrient amount is important for the diffuse nutrient supply from the dermis to the avascular epidermis, because the diffusion efficiency is determined by the concentration gradient of nutrients in blood vessels and the rate of perfusion (Mülling, 2000; Winkler, 2005). Supplementing solely Zn may be less effective to influence (*i.e.* improve) the diffusion of nutrients or the diffusion of Zn alone. This corresponds with the positive effects in sow studies supplementing a combined micromineral supplement.

Another interfering factor may be an aquadate claw quality. If the claw quality is adequate, the impact of nutrition and/or other predisposing factors to improve the quality remains low. In the longitudinal sow study (Chapter 6b), the mean claw lesion score was low, indicating a healthier claw. This may have hampered the effect of supplemental Zn in this study but also in other studies (Table 7.2).

Finally, the measurements used to determine claw quality may differ between studies and may not be specific, sensitive or repeatable enough. Some measurements are more subjective than others; claw lesion scoring relies on a subjective assessment, while measuring claw conformation with a digital calliper may be more objective. Therefore, observer bias may be present (Tuyttens *et al.*, 2014). No studies validated these measurements and it is not known which measurements reflect differentiations in claw quality when dietary interventions are evaluated. Some measurements have shown effects whereas others did not, making it difficult to draw conclusions. Including a wider range of claw quality measurements did not elucidate the role of Zn in maintaining claw quality.

Table 7.2. Effects of dietary zinc supplementation or combined with other micromineral supplementations (Cu, Mn, Co, Se) on claw quality in production animals.

Species	Dietary intervention	Micromineral source		Response*	Reference
Piglets	Zn	Level	+ / =	+ CC (claw width, toe height, claw volume, claw horn size), GW = Net horn growth and histology	Chapter 6a
Sows	Zn	Level	+ / =	+ CL (heel horn overgrowth and erosion, skin lesions), CC (heel height) and histology (distance dermal papillae heel horn) = remaining variables CL, CC, GW, net horn growth, hoof Zn concentration, histology, MCC	Chapter 6b
Sows†	Zn, Cu, Mn	OZ versus IZ	+	CL (heel horn erosion)	Aae, 2008
Sows†	Zn, Cu, Mn	OZ versus IZ	+	CL (vertical wall cracks)	Anil *et al.*, 2010a
Sows†	Zn, Cu, Mn	OZ versus IZ	+	CL and lameness	Anil *et al.*, 2010b
Sows	Zn, Cu, Mn	OZ versus IZ	+ / =	CC and minor influence on CL and lameness	Bradley, 2010
Sows	Zn, Cu, Mn	OZ versus IZ	+	CL, depending on herd	Da Silva *et al.*, 2010
Sows	Zn, Cu, Mn	OZ versus IZ	+ / =	+ CL (heel horn erosion) = CL (vertical wall cracks)	Anil, 2011
Calves	Zn	OZ versus IZ	=	Hoof Zn concentration	Rojas *et al.*, 1996
Calves	Zn	Level and Zn source	+ / =	+ Hoof Zn concentration for Zn sources = Hoof Zn concentration for Zn level	Wright and Spears, 2004
Dairy cows	Zn	Level organic Zn	+ / =	+ W = G	Randy *et al.*, 1985
Dairy cows	Zn	Level organic Zn	+	CL (foot rot, heel horn erosions, IDD, laminitis, sole ulcers, white line lesions)	Moore *et al.*, 1988
Dairy cows	Zn	OZ versus IZ	+ / =	+ CL (foot rot, heel cracks, IDD, texture) = GW	Moore *et al.*, 1989
Dairy cows	Zn, Cu, Mn, Co	OZ versus IZ	=	Lameness	Uchida *et al.*, 2001
Dairy cows	Zn, Cu, Mn, Co	OZ versus IZ	+	CL (heel erosion, white line, sole haemorrhages)	Ballantine *et al.*, 2002

Table 7.2. *Continued*

Species	Dietary intervention	Micromineral source		Response	Reference
Dairy cows	Zn, Cu, Mn, Co	Organic Zn	+	CL (double sole, DD, white line, sole haemorrhages, sole ulcers)	Nocek *et al.*, 2002
Dairy cows	Zn, Cu, Mn, Co	OZ versus IZ	+	CL (sole ulcers and lesions)	Ferguson *et al.*, 2004
Dairy cows	Zn, Cu, Mn	OZ versus IZ	+	CL	Drendel *et al.*, 2005
Dairy + beef cattle	Zn, Cu, Se	-	+	Lameness	Enjalbert *et al.*, 2006
Dairy cows	Zn, Cu, Mn, Co	Level and Zn source	+ / =	+ CL (heel erosion, white line separation) = CL (sole ulcers, sole haemorrhage, DD)	Nocek *et al.*, 2006
Dairy cows	Zn, Cu, Mn, Co	Level organic Zn	=	MCC (claw hardness)	Griffiths *et al.*, 2007
Dairy cows	Zn, Cu, Mn	OZ versus IZ	=	Lameness	Toni *et al.*, 2007
Dairy cows	Zn, Cu, Mn, Co	OZ versus IZ	+	CL (sole ulcers)	Siciliano-Jones *et al.*, 2008
Dairy cows	Zn	OZ versus IZ	=	MCC (claw hardness) and lameness	Cope *et al.*, 2009
Dairy cows	Zn	Level and Zn source	=	CL (sole and white line), CC, GW, lameness	Lethbridge, 2009
Dairy cows	Zn, Cu, Mn, Co	OZ versus IZ	=	Lameness	Hackbart *et al.*, 2010
Dairy cows	Zn, Cu, Mn	OZ versus IZ	=	CL	Formigoni *et al.*, 2011
Dairy cows	Zn, Cu, Mn, Se	OZ versus IZ	=	Lameness	Karkoodi *et al.*, 2012
Dairy cows	Zn	Level organic Zn	+ / =	+ CL (heel erosions, sole avulsions, white line haemorrhages) = Lameness, leg conformation, hoof Zn concentration	Randhawa, 2012
Dairy cows	Zn, Cu, Mn	OZ versus IZ	+	MCC (claw hardness)	Zhao *et al.*, in press
Beef cattle	Zn	Level and Zn source	+ / =	+ Clinical status, histology (tubules of coronary band) = MCC (tensile strength)	Stern *et al.*, 1998

Table 7.2. *Continued*

Species	Dietary intervention	Micromineral source		Response	Reference
Steers	Zn	OZ versus IZ	+	CL (footrot)	Brazle, 1993
Bulls	Zn	Level and Zn source	+ / =	+ Histology (macroscopic- clinical claw examination) = MCC (tensile strength), hoof Zn concentration	Kessler *et al.*, 2003
Young bulls	Zn	Level	+	Lameness	Fagari-Nobijari *et al.*, 2012
Lambs	Zn	OZ versus IZ	+ / =	+ CL (foot rot under dry conditions) = CL (foot rot under wet conditions	Cross and Parker, 1981
Lambs	Zn	OZ versus IZ	=	Hoof Zn concentration	Rojas *et al.*, 1995
Broilers	Zn	Level	=	Leg scores (hock joint deformities)	Sunder *et al.*, 2008

OZ, organic Zn sources; IZ, inorganic Zn sources; CL, claw lesion scoring; CC, claw conformation; GW, horn growth and wear; G, horn growth; W, horn wear; Histology, histological claw characteristics; MCC, mechanical claw characteristics; DD, digital dermatitis; IDD, interdigital dermatitis.

[*] Responses of claw quality measurements on the dietary intervention are represented as response (+), no response (=) or a response dependent on type of measurements (+ / =). If a response is dependent on measurement this is indicated under measurements for both findings. The type of measurements are indicated between brackets.

[†] Inorganic Zn source partially substituted by organic Zn source.

Limitations in experimental conditions related to claw quality

The dietary Zn intervention studies (Chapter 6a and 6b) offer valuable insights, but a role of Zn in claw quality was not identified for sows. In this context, some limitations need to be considered.

Some claw conformation measurements differed initially (4 weeks of age) between the two dietary treatment groups in weaned piglets (Chapter 6a). This may hamper sound conclusions on whether differences present at 9 weeks of age originated from the initial differences or from the dietary Zn concentration. However, some of the initial differences were unaffected, while other differences disappeared and new differences occurred over time. It remains unclear why these initial differences were present, because the piglets were randomly allocated to the treatment groups, and to what extent they have biased the results.

Unfortunately, the dietary Zn concentration of the non-supplemented lactation diet in the longitudinal sow study (Chapter 6b) was higher than formulated and equalled practical dietary Zn concentrations. A possible impact of the non-supplemented gestation diet may have been biased by the high Zn concentration during lactation (fed from 1 week before parturition until weaning, n= 5 weeks). However, the extent is not known. Feeding a non-supplemented gestation diet did not affect claw quality.

Clinically healthy non-lame gilts started in the longitudinal study (Chapter 6b) and the mean claw lesions score throughout the experiment was close to 40 mm (upper threshold for healthy claws). It is therefore worth questioning whether dietary Zn concentration could have improved the already adequate claw quality within the period studied. If sows with more severe claw lesion scores (or higher parity sows, who seem to have more severe claw lesion scores (Pluym *et al.,* 2011; Calderón Díaz *et al.,* 2014)) were included, then the effect of dietary Zn concentration might have been different. Obviously, there were sows with higher (more severe) lesions scores throughout the experiment. However, the effect of dietary Zn concentration on these maximal scores (68± 2.7 (SE) mm, range between 43 and 88) for claw lesions was not different from the effect found on the mean claw lesion score. Furthermore, in practice, most sows with severe claw lesion or lameness scores are culled before the 4th parity (Pluym *et al.,* 2013b; Calderón Díaz *et al.,* 2014), indicating that the use of primiparous sows up to three reproductive cycles covered the problematic time period for claw lesion and lameness development under practical conditions.

The effect of dietary Zn concentration in sows (Chapter 6b) was determined using three dietary treatment groups with a mixture of inorganic and organic Zn. There was no treatment group where Zn was added either as inorganic or as organic source. This may have influenced the response, assuming that organic Zn is better available than inorganic Zn sources, more effects could be expected if one diet was supplemented with only organic Zn. We could not confirm this assumption

as Zn source did not influence Zn bioavailability in our study (Chapter 5), and also other studies in pigs found no positive effect of Zn source on Zn bioavailability (Lee *et al.*, 2001; Case and Carlson, 2002; van Heugten *et al.*, 2003; Carlson *et al.*, 2004; Jongbloed, 2010; Siebert *et al.*, 2010; Paulicks *et al.*, 2011). Furthermore, we wanted to represent practical conditions, in which Zn is mostly added inorganically or inorganically combined with organic Zn.

Claw lesion scoring is a subjective observation compared to claw conformation measurements. Multiple (n= 3) observers scored the claws for claw lesions, alternately. This may have influenced the scores and may have increased the variation. However, the three observers were trained similarly and matched in claw scoring. Above, animals of different treatment groups were scored randomly spread over the day.

Furthermore, the scoring system we have used for claw lesion scoring was adapted from the scoring guide of Zinpro Corporation and Wageningen University (*i.e.* Zeugenklauwencheck) to include more types of claw lesions and to be able to differentiate extensively in severity. This tVAS scoring scale deviates from scoring systems using ordinal scales in other studies and make comparison between studies difficult. Yet, the categories itself of our tVAS scoring scale can be used as ordinal scale and allows additionally to distinguish in the severity of claw lesions within categories. In our studies, we scored clean front and hind claws in the sow chute (© Zinpro Corporation, Eden Prairie, MN, USA) during gestation and lactation. In most studies, only the hind claws are scored in the farrowing crate, with the sow lying down. In the farrowing crate, it is difficult to clean the claws and vision is not always clear. It is possible that only severe lesions have been detected in other studies.

Perspectives for future research

This thesis describes extensive studies on Zn status assessment and the effect of dietary Zn on Zn status and claw quality in pigs, attempting to elucidate the role of Zn in claw quality. The extensive research that has been done in this thesis shows that even more research is needed in order to provide adequately estimated Zn requirements and nutritional preventive strategies for claw lesions in the near future:

- Phase within the reproductive cycle influenced both Zn status biomarkers and claw quality measurements. This indicates that physiological processes within the sow are changed or adjusted during reproduction to maintain homeostasis. The underlying mechanisms and concomitant processes that incite these physiological differences are unclear for pigs. It would be worthwhile to study these mechanisms involved during reproduction. The gathered information will be useful for better understanding Zn metabolism.

- Zinc and protein source did not influence Zn status or Zn bioavailability in sows during late gestation. The dietary Zn concentration equalled conventional concentrations, which are higher than the recommendations, because practical conditions were mimicked. However, the outcome may differ when Zn inclusion levels to the diet are lower, other Zn and protein sources or combinations are used, or when more sensitive biomarkers are included. Furthermore, sows during late gestation may respond differently to this dietary strategy compared to other reproductive phases, such as during lactation were the Zn requirements are also high. Future studies could assess the (*in vitro*) hypothesis of increased Zn bioavailability under other experimental settings that might contribute to a dietary strategy to minimise Zn excretion and consequently reduced environmental pollution.
- The age of pigs and phase within the reproductive cycle influence the effect of Zn on claw quality. Effects early in life may differ from later in life. Studies should be performed to identify the conditions where Zn can improve claw quality from birth throughout reproduction. This new information will contribute to the understanding of the effects of insufficient or excessive dietary Zn concentrations on claw quality throughout the complete life span of pigs and may identify critical periods.
- This thesis did not define the potential interactions and combined effects of Zn with other (micro)minerals. Minerals do influence the absorption or excretion of other minerals at insufficient or excessive dietary concentrations. The dietary concentrations of other minerals equalled recommended concentrations in all performed experiments. The dietary Zn concentration did not influence Zn status biomarkers and most claw quality measurements in sows. However, the effect of Zn may be different when dietary concentrations of other minerals vary. Future studies should validate the minor impact of dietary Zn concentration on Zn status and claw quality when dietary concentrations of other minerals fluctuate.

Main conclusion

Assessing Zn status to monitor the impact on health issues such as claw health requires a careful selection of biomarkers and one has to consider the feed-independent fluctuations of Zn status biomarkers throughout the reproductive cycle. Within the tested range of dietary Zn concentrations, the Zn inclusion level, Zn source, and protein source did not influence Zn status biomarkers in sows. Likewise, claw quality seemed not to be affected in sows by the Zn inclusion level of the gestation diet, although periods of lower Zn status (decreased plasma Zn and serum MT concentration) during reproduction were present. Claw quality varied considerably throughout the reproductive cycle, indicating that phase within the reproductive cycle is as important for claw

quality as it is for Zn status biomarkers. In weaned piglets, dietary Zn concentration seems to be important, affecting claw quality if Zn was not supplemented to the diet, yet, more research is warranted.

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Summary

Lameness and claw lesions in pigs are a multifactorial disorder and a major concern for the animals' welfare and farm profitability. Almost all sows have one or more claw lesions, varying in type and severity. To prevent claw lesions in pigs, the quality of the claw should be optimal to maintain adequate horn production. The adverse impact of predisposing factors, such as problems with floor type, management and nutrition needs to be minimised.

A comprehensive review (Chapter 1) revealed that nutrition influences claw quality by the diffuse nutrient supply from the dermis to the avascular epidermis. This diffuse nutrient supply could be interrupted. Consequently, claw quality is negatively influenced and the claw becomes susceptible to developing claw lesions. Some dietary components, such as biotin and sulphur containing amino acids, contribute positively to claw quality. Zinc, as an essential micromineral, seems to influence the processes required for horn production, yet the precise role is unclear. Zinc is important for multiple body functions. The metabolism of Zn is tightly regulated to maintain homeostasis and to ensure that all functions pursue. The assessment of Zn status is, however, difficult. Therefore, this thesis focussed on Zn status assessment and the role of dietary Zn on Zn status and claw quality in pigs (Chapter 2).

In the first study (Chapter 3), the suitability of biomarkers for Zn status assessment to diagnose Zn deficiency or to determine Zn requirements and Zn availability was evaluated for production animals. Responses to dietary Zn concentration differ between biomarkers and not all biomarkers are suitable for all study objectives. Dietary Zn intake is preferred as additional biomarker to plasma Zn concentration or daily growth as response criterion. Body tissues and plasma alkaline phosphatase (ALP) seem less suitable, and metallothionein (MT) as biomarker requires further research. Biomarkers therefore need to be selected based on the particular research question.

The concentration of some suitable biomarkers may be influenced by reproduction. In order to understand clearly the physiological variations of biomarkers throughout the reproductive cycle of sows, we performed an observational study with the most suitable biomarkers for Zn status assessment (Chapter 4). Five primiparous and 10 multiparous sows were followed as one group for one reproductive cycle. Blood samples collected at regular intervals during the reproductive cycle were analysed for plasma Zn, serum MT, serum ALP, and serum albumin concentration. Fluctuations were found for each biomarker throughout the reproductive cycle ($P<0.050$), independently of parity, but the observed patterns differ among the biomarkers.

As phase within the reproductive cycle interferes with Zn status biomarkers, dietary Zn concentration, Zn source and protein source may influence Zn status as well. We tried to identify the impact of Zn source and protein source in sows during late gestation (Chapter 5). Sows (n= 56) at the end of gestation (d86) were fed a diet that differed in the combination of Zn and protein

source for 20 days. An organic or inorganic Zn source was supplemented to soybean meal or hydrolysed feather meal. Plasma Zn concentration tended to decrease from d1 to d20 (P=0.053), whereas serum MT concentration increased (P<0.001). No interaction was found between Zn and protein source for plasma Zn (P=0.158) and serum MT concentration. Neither Zn nor protein source affected the apparent faecal Zn absorption (P=0.360 and P=0.527, respectively) or faecal Zn concentration (P=0.442 and P=0.385, respectively). The nutrient digestibility was lower in sows fed diets that include the hydrolysed feather meal (P<0.001). In this study, Zn source and protein source were no major factors influencing Zn status.

The impact of dietary Zn concentration on Zn status biomarkers and claw quality was studied in weaned piglets (Chapter 6a) and sows (Chapter 6b). Weaned piglets (n= 24, 4 weeks of age) were fed a non-supplemented diet (42 mg Zn/kg diet, Zn originates from the ingredients only) or Zn-supplemented diet (106 mg Zn as ZnO/kg diet) for 5 weeks. Within 5 weeks, plasma Zn concentration was 24.2 μg/dL lower for non-supplemented piglets (P=0.003). In addition, horn growth and wear was lower in non-supplemented piglets (P=0.044 and P<0.001, respectively). Although, claw conformation differed, histological claw characteristics were not different between the two dietary treatment groups (P>0.100) (Chapter 6a). In the longitudinal study on six groups of sows (n= 131 at the start), sows within each group were randomly divided into a non-supplemented, 50 or 100 mg Zn/kg supplemented dietary treatment group (Chapter 6b). The sows were followed for three reproductive cycles and group housed on concrete or rubber top-layer floors during d28 and d108 of gestation. In this longitudinal study, serum MT concentrations and concentrations of Zn in plasma, liver, and bone did not differ between dietary treatment groups (P=0.771, P=0.125, P=0.717 and P=0.262, respectively). This indicates that all sows were able to maintain Zn homeostasis. However, (reproductive) performance was lower for the 100 mg Zn/kg supplemented sows (P<0.010). The fluctuations observed for plasma Zn and serum MT concentrations in chapter 4 were confirmed in this study. For claw quality, dietary Zn concentration seems to play a minor role, especially compared to effects found for floor type. Only heel horn erosion scores were better for the 100 mg Zn/kg supplemented sows at d50 of the reproductive cycle (P=0.014), but the difference of 4.2 mm was small and might be irrelevant. Some differences were found for claw conformation but no systematic differences were found over the wide range of measurements included in this study.

In conclusion, assessing Zn status to monitor the impact on claw quality requires a careful selection of biomarkers. Fluctuations of Zn status biomarkers throughout the reproductive cycle interfere with the observed concentrations of biomarkers, independently of dietary Zn concentration. To interpret the observed Zn status, phase within the reproductive cycle has to be taken into account.

Within the tested range of dietary Zn concentrations, the Zn inclusion level, Zn source, and protein source did not influence Zn status biomarkers in sows. Likewise, claw quality seemed not to be affected in sows by the Zn inclusion level of the gestation diet, although periods of lower Zn status (decreased plasma Zn and serum MT concentration) during reproduction were present. Claw quality varied considerably throughout the reproductive cycle, indicating that phase within the reproductive cycle is as important for claw quality as it is for Zn status biomarkers. In weaned piglets, dietary Zn concentration seems to be important, affecting claw quality if Zn was not supplemented to the diet, but more research is warranted.

Future research should focus on performing studies to identify the physiological mechanisms involved during reproduction, which controls and directs the fluctuations of Zn status biomarkers. This will improve the understanding of Zn metabolism. Other experimental conditions and biomarkers can be implemented to evaluate the effect of Zn and protein source on Zn status biomarkers and Zn bioavailability. Furthermore, the condition where Zn can improve claw quality should be identified from birth throughout reproduction. Lastly, future studies should validate the minor role of dietary Zn concentration on Zn status and claw quality when dietary concentrations of other mineral fluctuate.

Samenvatting

Kreupelheid en klauwproblemen bij varkens zijn multifactoriële aandoeningen en zijn een belangrijk aandachtspunt voor het dierenwelzijn en de rentabiliteit van het bedrijf. Bijna alle zeugen hebben één of meer klauwproblemen, variërend in type en ernst. Om klauwproblemen bij varkens te voorkomen, moet de kwaliteit van de klauw gehandhaafd blijven zodat de hoornproductie optimaal blijft. De nadelige gevolgen van predisponerende factoren, zoals problemen met het vloertype, management, en voeding moeten geminimaliseerd worden.

Uit de review (Hoofdstuk 1) is gebleken dat voeding de klauwkwaliteit kan beïnvloeden. De voedingsstoffen worden door diffusie vanuit de dermis naar de avasculaire opperhuid verplaatst. Deze diffuse toevoer van voedingsstoffen kan onderbroken worden. Daardoor wordt de klauwkwaliteit negatief beïnvloed en wordt de klauw gevoelig voor de ontwikkeling van klauwproblemen. Sommige voedingsbestanddelen zoals biotine en zwavelhoudende aminozuren dragen positief bij aan klauwkwaliteit. Zink, als essentieël micromineraal, lijkt belangrijk te zijn voor de processen die nodig zijn voor hoornproductie, maar de precieze rol is onduidelijk. Zink is belangrijk voor veel lichaamsfuncties. Het metabolisme van Zn is sterk gereguleerd voor homeostase, zodat alle functies die Zn nodig hebben voortgezet worden. Het bepalen van Zn status is echter moeilijk. Dit proefschrift verdiept zich in het bepalen van Zn status en de rol van Zn concentratie in het voeder op Zn status en klauwkwaliteit bij varkens (Hoofdstuk 2).

In de eerste studie (Hoofdstuk 3) werden biomarkers voor Zn status beoordeeld op de geschiktheid om Zn deficiëntie te diagnosticeren of om de Zn behoefte en beschikbaarheid te bepalen bij productiedieren. De reactie van biomarkers op verschillende Zn concentraties in het voeder verschilde tussen biomarkers en niet alle biomarkers zijn geschikt voor alle doelstellingen. De voederinname van Zn wordt bij voorkeur als aanvullende biomarker gebruikt bij plasma Zn concentratie of dagelijkse groei. Lichaamsweefsels en plasma alkalisch fosfatase (ALP) lijken minder geschikt en metallothioneïne (MT) als biomarker verdient verder onderzoek. Biomarkers moeten gekozen worden op basis van de studie-specifieke doelstelling.

De concentratie van een aantal biomarkers kan beïnvloed worden door reproductie. Om een duidelijk inzicht te krijgen in de variaties van biomarkers gedurende de reproductieve cyclus van zeugen, voerden wij een observationele studie uit met de meest geschikte biomarkers voor Zn status (Hoofdstuk 4). Vijf primipare en 10 multipare zeugen werden opgevolgd gedurende een reproductieve cyclus. Bloedmonsters werden met regelmatige interval verzameld en geanalyseerd op plasma Zn, serum MT, serum ALP en serum albumine concentratie. Voor elke biomarker werden fluctuaties waargenomen gedurende de reproductieve cyclus ($P<0{,}050$), onafhankelijk van pariteit, maar de waargenomen patronen verschilden tussen de biomarkers.

Fase in de reproductieve cyclus beïnvloedde de concentraties van biomarkers voor Zn status. Echter kunnen de Zn concentratie in het voeder, Zn bron en eiwitbron de Zn status ook beïnvloeden. We hebben geprobeerd om de impact van Zn bron en eiwitbron bij zeugen te identificeren tijdens late dracht (Hoofdstuk 5). Zeugen (n= 56) aan het eind van de drachtperiode (d86) werden gedurende 20 dagen gevoederd met een voeder dat verschilde in de combinatie van Zn- en eiwitbron. Een organische of anorganische Zn bron werd toegevoegd aan een voeder met sojameel of gehydrolyseerd verenmeel. Plasma Zn concentratie leek te dalen van d1 tot d20 (P=0,053), terwijl de serum MT concentratie toenam (P<0,001). Er werd geen interactie gevonden tussen Zn- en eiwitbron voor plasma Zn (P=0,158) en serum MT concentratie. Noch Zn bron noch eiwitbron beïnvloedde de schijnbare faecale Zn absorptie (P=0,360 en P=0,527, respectievelijk) of de faecale Zn concentratie (P=0,442 en P=0,385, respectievelijk). De verteerbaarheid van nutriënten was lager bij zeugen gevoederd met gehydrolyseerd verenmeel (P<0,001). Uit deze studie bleek dat noch de Zn bron noch eiwitbron belangrijke factoren waren die de Zn status beïnvloedden.

De impact van Zn concentratie in het voeder op de biomarkers voor Zn status en klauw kwaliteit werd onderzocht bij gespeende biggen (Hoofdstuk 6a) en zeugen (Hoofdstuk 6b). Gespeende biggen (n= 24, 4 weken leeftijd) kregen gedurende 5 weken een niet-gesupplementeerd voeder (42 mg Zn/kg voeder, Zn afkomstig van de ingrediënten) of Zn gesupplementeerd voeder (106 mg Zn als ZnO/kg voeder). Binnen 5 weken was de plasma Zn concentratie 24,2 ug/dL lager voor niet-gesupplementeerde biggen (P=0,003). Ook hoorngroei en -slijtage waren lager voor deze groep biggen (P=0,044 en P<0,001, respectievelijk). Hoewel klauwconformatie verschilde, weken histologische kenmerken niet af voor de twee voedergroepen (P>0,100) (Hoofdstuk 6a). In de longitudinale studie bij zes groepen zeugen (n= 131 bij aanvang van de studie), werden zeugen binnen elke groep willekeurig verdeeld in een niet-gesupplementeerde, 50 of 100 mg Zn/kg voeder gesupplementeerde voedergroep (Hoofdstuk 6b). De zeugen werden 3 reproductieve cycli opgevolgd en gehuisvest in groepen van d28 tot d108 tijdens dracht op een vloer met beton of beton met rubber toplaag. In deze longitudinale studie verschilde serum MT concentratie en Zn concentratie in plasma, lever en bot Zn concentratie niet tussen de voedergroepen (P=0,771, P=0,125, P=0,717 en P=0,262, respectievelijk). Zink homeostase kon gehandhaafd worden door alle zeugen. Echter, de conditie voor de 100 mg Zn/kg gesupplementeerde zeugen was lager (P<0,010). De waargenomen fluctuaties van plasma Zn en serum MT concentraties in hoofdstuk 4 werden bevestigd in deze studie. Voor klauwkwaliteit lijkt Zn concentratie in het voeder een ondergeschikte rol te spelen, vooral in vergelijking met de effecten gevonden voor vloertype. Alleen de scores voor balhoorn erosie waren beter voor de 100 mg Zn/kg gesupplementeerde zeugen op d50 van de cyclus (P=0,014), maar het verschil van 4,2 mm is klein en mogelijk

irrelevant. Ondanks een aantal verschillen tussen voedergroepen voor klauwconformatie werden geen systematische verschillen gevonden.

Tot slot, om de impact op Zn status en klauwkwaliteit te bepalen dienen biomarkers zorgvuldig geselecteerd te worden. Fluctuaties van Zn status biomarkers tijdens de reproductieve cyclus beïnvloeden de gevonden concentraties van de biomarkers, onafhankelijk van de Zn concentratie in het voeder. Bij het interpreteren van Zn status zal daarom rekening gehouden moeten worden met de fase in de reproductieve cyclus. De gebruikte Zn concentraties in het voeder, Zn bron en eiwitbron beïnvloeden de Zn status biomarkers bij zeugen niet. Klauwkwaliteit leek ook niet te worden beïnvloed door de Zn concentratie in het voeder, hoewel tijdens de reproductie periodes van een lagere Zn-status (verlaagde plasma Zn en serum MT concentraties) gevonden werden. Klauwkwaliteit variëerde tijdens de reproductieve cyclus, waardoor fase in de reproductieve cyclus belangrijk is voor zowel Zn status als voor klauwkwaliteit. Bij jonge dieren lijkt Zn concentratie in het voeder belangrijk voor klauwkwaliteit, maar meer onderzoek is benodigd. Bij gespeende biggen werd zowel plasma Zn concentratie als klauwkwaliteit negatief beïnvloed als geen Zn werd toegevoegd aan het voeder.

Toekomstige studies kunnen het beste gericht worden op onderzoek naar de fysiologische mechanimsen die de fluctuaties van Zn status biomarkers tijdens de reproductieve cyclus controleren en aansturen. Dit verbetert het inzicht over het metabolisme van Zn. Om het effect van Zn bron en eiwitbron op de biomarkers voor Zn status en biobeschikbaarheid verder te onderzoeken, zouden andere experimentele condities en biomarkers gebruikt kunnen worden. Bovendien zouden de omstandigheden waarbij Zn de klauwkwaliteit kan verbeteren geïdentificeerd kunnen worden vanaf de geboorte tot reproductie. Tenslotte kunnen toekomstige studies de geringe rol van Zn op Zn status en klauwkwaliteit valideren wanneer concentraties van andere mineralen in het voeder variëren.

About the author

Curriculum vitae

Miriam M.J. van Riet werd geboren op 13 september 1987 te Mierlo (Nederland) en behaalde in 2004 haar HAVO diploma met profiel Natuur & gezondheid aan het Jan van Brabant college te Helmond. In 2004 startte Miriam de opleiding Dier- en veehouderij aan de Internationale Agrarisch Hogeschool Larenstein te Deventer en studeerde af in 2008 in de richting Diergezondheid en –welzijn. Tijdens deze studie maakte Miriam kennis met kwantitatief onderzoek naar histologische methodieken en morfologie van kraakbeen bij paarden en onderzocht ze de invloed van krachtvoer op de passagesnelheid en retentie tijd in het maagdarmkanaal van paarden met behulp van 3 markers. Miriam vervolgde haar studie door de Master in Animal Science te volgen aan de universiteit van Wageningen te Wageningen en zich te specialiseren in Diervoeding, waarvan ze in 2010 afstudeerde. Tijdens haar afstudeeropdrachten lag de focus op klauwproblemen bij melkkoeien met het gebruik van een nieuw middel in voetbaden als preventie maatregel, maar werd ook de verhouding tussen krachtvoer en ruwvoer op de verteerbaarheid van nutriënten onderzocht bij paarden. Vervolgens startte Miriam in februari 2011 haar doctoraatstudie aan het Instituut voor Landbouw en Visserij onderzoek (ILVO) te Melle in samenwerking met Universiteit Gent (België), waarbij kennis van eerdere studies samenvielen om het effect van Zn in voeding op Zn status en klauwproblemen bij varkens te onderzoeken.

Miriam M.J. van Riet was born on September 13, 1987 in Mierlo (The Netherlands). In 2004, she graduated from high school (Jan van Brabant College, Helmond, The Netherlands) and started her study Animal husbandry at the University of Applies Sciences (Van Hall Larenstein, Deventer, The Netherlands). Miriam completed this study successfully in 2008 with the specialisation Animal health and welfare. For this specialisation, she focussed on histological methods and the morphology of articular cartilage in horses. Furthermore, she investigated the influence of concentrate on the passage rate and rentention time in the gastrointestinal tract of horses using the marker method. Miriam continued her studies in 2008. She started the master study Animal Sciences at Wageningen Univeristy (The Netherlands) with the specialisation Animal Nutrition and graduated in 2010. During this study, Miriam investigated the effect of a new acidified ionised copper solution, as preventive measure, on digital dermatitis in dairy cows and determined the effect of different concentrate: roughage ratios on nutrient digestibility in horses. Miriam started her PhD research at the Institute for Agricultural and Fisheries Research (ILVO) in collaboration with Ghent University (Belgium) in 2011. She focused on the assessment of zinc status and the impact of dietary zinc on zinc status and claw quality in pigs.

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List of publications

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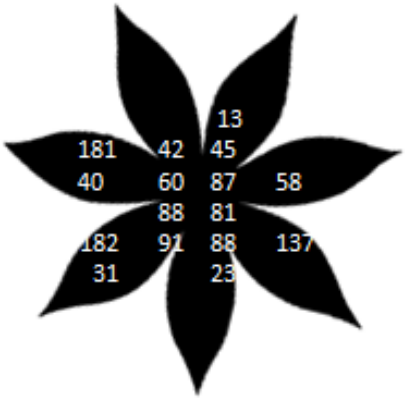

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Or in other words: thanks to sows & piglets

"Never lose a holy curiosity"

Albert Einstein

"Now this is not the end
It is even not the beginning of the end
But it is perhaps the end of the beginning"

Winston Churchill